

AD-A180 183

DTIC FILE COPY

(3)

FRESHWATER CYANOBACTERIA (BLUE-GREEN ALGAE) TOXINS:  
ISOLATION AND CHARACTERIZATION

AD \_\_\_\_\_

ANNUAL REPORT

BY

WAYNE W. CARMICHAEL, PH.D.  
ASSOCIATE PROFESSOR

DTIC  
ELECTE  
MAY 15 1987  
S D

OCTOBER, 1985

SUPPORTED BY  
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
FORT DETRICK, FREDERICK, MARYLAND 21701-5012

CONTRACT NO. DAMD17-84-C-4156

DEPARTMENT OF BIOLOGICAL SCIENCES  
WRIGHT STATE UNIVERSITY  
DAYTON, OHIO 45435

Approved for Public Release;  
Distribution Unlimited

The findings in this report are not to be construed as an  
official Department of the Army position unless so designated by  
other authorized documents.

20030127037

## REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION			1b. RESTRICTIVE MARKINGS		
2a. SECURITY CLASSIFICATION AUTHORITY			3. DISTRIBUTION/AVAILABILITY OF REPORT Approved for Public Release; Distribution Unlimited		
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE					
4. PERFORMING ORGANIZATION REPORT NUMBER(S)			5. MONITORING ORGANIZATION REPORT NUMBER(S)		
6a. NAME OF PERFORMING ORGANIZATION Dept. of Biological Sciences Wright State University		6b. OFFICE SYMBOL (If applicable)		7a. NAME OF MONITORING ORGANIZATION U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID)	
6c. ADDRESS (City, State, and ZIP Code) Dayton, OH 45435				7b. ADDRESS (City, State, and ZIP Code) Ft. Detrick, Frederick, MD 21701	
8a. NAME OF FUNDING/SPONSORING ORGANIZATION U.S. Army Medical Res. & Dev- elopment		8b. OFFICE SYMBOL (If applicable)		9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD17-84-C-4156	
8c. ADDRESS (City, State, and ZIP Code) Ft. Detrick, Frederick, Md. 21701		10. SOURCE OF FUNDING NUMBERS			
		PROGRAM ELEMENT NO.		PROJECT NO.	TASK NO.
					WORK UNIT ACCESSION NO.
11. TITLE (Include Security Classification) Freshwater Cyanobacteria (blue-green algae) Toxins: Isolation and Characterization					
12. PERSONAL AUTHOR(S) Wayne W. Carmichael					
13a. TYPE OF REPORT Annual		13b. TIME COVERED FROM 8/15/84 TO 8/14/85		14. DATE OF REPORT (Year, Month, Day) October 1985	
15. PAGE COUNT					
16. SUPPLEMENTARY NOTATION					
17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)		
FIELD	GROUP	SUB-GROUP	Biotoxins, Blue-green Algae Toxins, Cyanobacteria Toxins		
19. ABSTRACT (Continue on reverse if necessary and identify by block number) Biotoxins of freshwater blue-green algae (cyanobacteria) are currently classed as being either hepatotoxic peptides or neurotoxic alkaloids. This study is concerned with the examination of toxins from the species <u>Anabaena flos-aquae</u> , <u>Aphanizomenon flos-aquae</u> , <u>Microcystis aeruginosa</u> and <u>Oscillatoria agardhii</u> . Areas of investigation include: 1) Culture methods for toxic freshwater cyanobacteria, 2) Extraction and purification of toxins, 3) Chemical analysis of isolated toxins, 4) Toxicology and pharmacological effect, 5) Continued field studies of toxic bloom occurrence, isolation and culture and testing of new toxic strains.					
20. DISTRIBUTION/AVAILABILITY OF ABSTRACT <input type="checkbox"/> UNCLASSIFIED/UNLIMITED <input type="checkbox"/> SAME AS RPT <input type="checkbox"/> DTIC USERS			21. ABSTRACT SECURITY CLASSIFICATION		
22a. NAME OF RESPONSIBLE INDIVIDUAL			22b. TELEPHONE (Include Area Code)		22c. OFFICE SYMBOL

**FRESHWATER CYANOBACTERIA (BLUE-GREEN ALGAE) TOXINS:  
ISOLATION AND CHARACTERIZATION**

AD \_\_\_\_\_

**ANNUAL REPORT**

**BY**

**WAYNE W. CARMICHAEL, PH.D.  
ASSOCIATE PROFESSOR**

**OCTOBER, 1985**

**SUPPORTED BY  
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND  
FORT DETRICK, FREDERICK, MARYLAND 21701-5012**

**CONTRACT NO. DAMD17-84-C-4156**

**DEPARTMENT OF BIOLOGICAL SCIENCES  
WRIGHT STATE UNIVERSITY  
DAYTON, OHIO 45435**

**Approved for Public Release;  
Distribution Unlimited**

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

## SUMMARY

This annual report covers work in progress on "Freshwater Cyanobacteria (blue-green algae) Toxins: Isolation and Characterization". The first part of the report updates review material on toxins of freshwater cyanobacteria (See also Appendix D). The second part details studies covered under this contract as described in the contract workscope. The last part describes efforts at scaling up production of selected toxins for detailed structure/function and detection studies. This is part of a supplement to the original contract which was implemented in the third quarter of this current report period. The workscope areas include: 1) Development of culture methods for neuro and hepatotoxin producing strains of freshwater cyanobacteria. This work has centered on implementation of fermenter systems designed for semi-continuous harvesting of algal cells, in addition to optimization of culture conditions for control of toxin production. 2) Extraction, purification and analysis of neurotoxins and hepatotoxins. This work has centered on purification and analysis of cyclic peptide toxins of Microcystis aeruginosa and Anabaena flos-aquae. A manuscript submitted for publication on this topic is included in this report. 3) Toxicology and pharmacology of isolated toxins. Work has involved the cellular effects on liver of cyclic peptide toxins. An electron microscope study is in progress on the organelle/membrane effects of lethal and sublethal toxin levels with time. Another study involves detailing the enzyme kinetics and membrane ion effects of a new anticholinesterase compound. 4) Collaborative studies to investigate new occurrences of toxic blue-green algae and to isolate, culture, and examine new toxic species. This work has resulted in the examination and isolation of new toxic isolates of Microcystis aeruginosa from Wisconsin; Anabaena flos-aquae from South Dakota; and Oscillatoria agardhii from Norway.

Accession For	
NTIS CRA&I	<input checked="" type="checkbox"/>
DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	



## FOREWARD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of The Institute of Laboratory Animal Resources, National Research Council. (DHEW Publication No. (NIH) 78-23, Revised 1978).

## TABLE OF CONTENTS

Cover Page. . . . .	1
Report Documentation Page . . . . .	ii
Title Page. . . . .	1
Summary . . . . .	2
Foreward. . . . .	3
Table of Contents . . . . .	4
Body. . . . .	5
Review of recent work on Freshwater Cyanobacteria Toxins. . . . .	5-16
Work in Progress Under Contract Workslope	
A. Culture Methods for Toxin Producing Strains of Freshwater Cyanobacteria . . . .	16
B. Extraction, Purification and Analysis of Neurotoxins and Hepatotoxins . . . . .	16
C. Collaborative Studies on New Toxic Cyanobacteria Blooms; Culture of Toxic Strains and Isolation of Toxins . . . . .	18
Literature Cited. . . . .	19
Appendixes. . . . .	24
A. Manuscript: Investigations of freshwater Cyanobacteria (blue-green algae) toxic peptides I. Isolation, purification and characterization of peptides from <u>Microcystis aeruginosa</u> and <u>Anabaena</u> <u>flos-aquae</u> . . . . .	23
B. Manuscript: Physiological Effect of a Peptide Toxin Produced by the Freshwater Cyanobacteria (Blue-green Algae) <u>Microcystis</u> <u>aeruginosa</u> strain 7820. . . . .	51
C. C.V.: Dr. Arun Dabholkar. . . . .	64
D. Toxin Data Base Profiles - Blue-green Algae (Cyanobacteria) . . . . .	69
Distribution Statement . . . . .	111

## BODY

### Review of recent work on freshwater Cyanobacteria toxins

#### 1. NEUROTOXINS

Known chemical groups of toxins from freshwater cyanobacteria include alkaloids and peptides. The alkaloid toxins are the most rapidly acting. They function as neurotoxins paralyzing peripheral skeletal muscles, then respiratory muscles, with death due to respiratory arrest occurring between a few minutes to a few hours. These toxins are produced by various strains and species of Anabaena and are generally referred to as anatoxins. Anatoxin-a (antx-a) is the only alkaloid toxin from this group which has been chemically characterized. It is a potent depolarizing neuromuscular blocking agent (Carmichael et al., 1975, 1979; Spivak et al., 1980). Chemically the compound is the secondary amine, 2-acetyl-9-azabicyclo[4.2.1]non-2-ene, and is isolated from the filamentous strain Anabaena flos-aquae NRC-44-1 (Huber, 1972; Devlin et al., 1977). Synthesis of antx-a has been done through a ring expansion of cocaine (Campbell et al., 1977) from 1,5-cyclooctadiene (Campbell et al., 1979), and by intramolecular cyclization between an iminium salt and a nucleophilic carbon atom (Bates and Rapoport, 1979).

Poisoning of domestic and wild animals by anatoxins comes from ingestion of the toxic cells and extracellular toxin from a water bloom. Given the LD<sub>50</sub> for intraperitoneal bioassay in mouse for purified anatoxin-a as 200 ug/kg body weight and the approximate oral lethal dose for certain animals (Carmichael et al., 1977; Carmichael, 1981) it is estimated that a lethal bolus of water bloom ranges from a few cubic centimeters to a few cubic decimeters, depending upon animal species, toxicity of the bloom, and amount of food material in the animal's gut. The presence of more than one toxin in the bloom can also result in different signs of poisoning and survival times.

Known toxins of Aphanizomenon flos-aquae are also neurotoxic alkaloids, generally referred to as aphantoxins. Sasner and his colleagues presented evidence that aphantoxin from water blooms of Aph. flos-aquae was similar to the paralytic shellfish poisons saxitoxin and neosaxitoxin (Sawyer et al., 1968; Jackim and Gentile, 1968; Alam et al., 1973, 1978; Alam and Euler, 1981; Sasner et al., 1981; Adelman et al., 1982). Current research with aphantoxin is centered on toxic strains isolated from a small pond near Durham, New Hampshire in 1980 by Carmichael, referred to as NH-1 and NH-5 (Carmichael, 1982; Ikawa et al., 1982; Mahmood and Carmichael, 1986a). The toxins of NH-5 are primarily neosaxitoxin (80%) and saxitoxin (15%). Depending on extraction procedure it appears that aphantoxin also contains

some precursors to neosaxitoxin and saxitoxin (Carmichael and Mahmood, 1984; Sasner et al., 1984, Mahmood and Carmichael, 1986a). At present the structures of the aphantoxins have not been determined but assuming that they are the same as PSPs, and all evidence to date supports this, they will have structures like those of saxitoxin and neosaxitoxin.

Detection methods for these toxins in water supplies have not been adequately developed but high-performance liquid chromatography (HPLC) is being used in some cases. Both Astrachan and Archer (1981) and Wong and Hindin (1982) have used HPLC for the detection of anatoxin-a. In our laboratory we routinely use HPLC to purify other neurotoxins of Anabaena and Aphanizomenon (Carmichael and Mahmood, 1984). These methods could be modified to detect low levels of the toxins in water reservoirs or recreational waters.

## 2. HEPATOTOXINS

The peptide toxins of the freshwater cyanobacteria are primarily found in various strains of Microcystis aeruginosa. These toxins are responsible for most poisonings by cyanobacteria since M. aeruginosa is more common in its worldwide distribution than the other toxigenic cyanobacteria, including Anabaena, Aphanizomenon, and Oscillatoria (Carmichael, 1981, 1982; Skulberg et al., 1984) are thought to produce peptide toxins, Microcystis peptides have been studied most. Toxic blooms have been found in ponds and lakes throughout the world including the U.S.A., Canada, the U.S.S.R., Europe, South Africa, India, Japan, the Middle East, and Australia. In temperate zones most toxic blooms occur in mid to late summer (Carmichael et al., 1985).

Rats and mice injected either intravenously or intraperitoneally with acutely toxic doses of the cells or toxin extract (LD<sub>50</sub> 25-100 ug/kg, intraperitoneally in mouse) die within 1 to 3 hr. Death is preceded by pallor or prostration, with episodes of unprovoked leaping and twitching. Upon necropsy, the animals show grossly enlarged livers engorged with blood, with the remainder of the carcass being exsanguinated. Liver weight is increased, and at death composes about 8 to 10% of body weight of mice as compared to about 5% in controls (Slatkin et al., 1983; Falconer et al., 1981; Theiss, 1984; Theiss and Carmichael, 1986). The blood content of livers of mice poisoned by Microcystis increases from 0.066 mm<sup>3</sup> g<sup>-1</sup> liver in controls to 0.543 mm<sup>3</sup> g<sup>-1</sup> for mice killed 45 min after toxin injection (Falconer et al., 1981).

Histological examination of the liver reveals extensive centrilobular hemorrhagic necrosis with loss of characteristic architecture of the hepatic cords. Transmission electron microscopic examination indicates that both hepatocytes and hepatic endothelial cells are destroyed. The only alterations



noted prior to cell rupture are slight mitochondrial and cell swelling. Damaged cells have extensive fragmentation and vesiculation of the membrane (Runnegar et al., 1981).

Gross and histological examination of intestine, heart, spleen, kidneys, and stomach show no consistent abnormalities; lungs are mildly congested with occasional patches of debris. Thrombi thought to contain platelets are found in the lungs of affected animals (Slatkin et al., 1983; Falconer et al., 1981). It is suggested that these thrombi may be a direct effect of the toxin and may secondarily cause the liver effects by creating sufficient pulmonary congestion to cause right heart failure which, in turn, could cause blood pooling and congestion in the liver (Slatkin et al., 1983). However, in time course studies, Falconer et al. (1981) reported that the pulmonary thrombi did not appear in histological preparations taken at 15 and 30 min after toxin injection and were present only in later preparations (while liver damage was noted as early as 15 min). This evidence, along with other evidence of effects on isolated hepatocytes and the rapid onset of the liver effects in vivo, have led other researchers to believe that the liver damage is a direct effect of the toxin on the hepatocyte membrane and that the immediate cause of death in acutely dosed animals is hemorrhagic shock (Falconer et al., 1981; Runnegar and Falconer, 1981; Theiss, 1984; Theiss and Carmichael, 1986). Occasionally, hemorrhages are noted in organs other than the liver (Ostensvik et al., 1981). This could possibly be due to coagulation problems associated with the liver damage.

The action of Microcystis toxin on isolated rat hepatocytes was investigated by Runnegar et al. (1981), Runnegar and Falconer (1981), and Foxall and Sasner (1981). In cell suspension, normal hepatocytes viewed under the scanning electron microscope show a rounded appearance with a surface covered with microvilli. Within 5 min of incubation with the toxin, obvious lumps can be seen on the cell surface, which increased with time to present a distorted lumpy cell without microvilli. These changes occur only in live cells and are found to be dose dependent, with the minimum concentration of toxin causing deformation being directly related to the LD<sub>50</sub> for the toxin. There is no difference in trypan blue exclusion by hepatocytes incubated with or without the toxin, the toxin causes no cell lysis, and there is no release of aspartate amino transferase (ASAT) into the medium. The toxin appears to be transported into the cell via the bile acid transporters in the cell membrane. Deformation of the cells by Microcystis toxin is blocked by addition of sodium deoxycholate to the medium. The blocking effect also shows a dose response, with increasing concentration of toxin requiring a higher bile acid concentration.

In another study by Grabow et al. (1982) the affect of Microcystis toxin was tested on isolated liver, lung, cervix,

ovary, and kidney cell cultures. Cells of all cultures were damaged or disintegrated after overnight incubation in the presence of Microcystis toxin. While no mechanism of action was proposed it as suggested that the toxin may act on cell membranes.

The effect on isolated hepatocytes of the presence and absence of calcium in the medium was also investigated (Runnegar and Falconer, 1982). It has been suggested by Farber and his co-workers that calcium entry into the cell is the final common step in the death of cells injured by membrane toxins and that cells can be protected by excluding calcium from the medium (Farber, 1981). Conversely, it has been reported by other researchers that toxic injury to hepatocytes is not dependent on calcium and in some cases calcium in the medium actually protects cells from injury by various toxins (Smith et al., 1981). Tests with Microcystis toxin and isolated hepatocytes revealed that the presence or absence of calcium in the medium makes no difference to the toxic effect (Runnegar and Falconer, 1982).

It was reported that toxic extracts from algal blooms would agglutinate red blood cells (Carmichael and Bent, 1981). However, Runnegar and Falconer reported no red blood cell agglutination with extracts isolated in their laboratory (Runnegar and Falconer, 1982).

The effects of the toxin on mouse liver slices, isolated mitochondria, and microsomes were investigated and no specific effects were noted (Runnegar and Falconer, 1981). The toxin did not significantly affect the incorporation of labeled leucine, uridine, or methylthymidine into trichloroacetic acid-insoluble precipitates from incubated mouse liver slices, indicating that it exerts no major effect on protein, RNA, or DNA synthesis. The toxin did not affect oxygen consumption of liver slices and only slightly increased that of isolated mitochondria. Measurements of glycogen degradation in liver slices incubated with toxin showed a variable increase in glycogen loss and glucose appearance in the incubation medium (Runnegar and Falconer, 1982).

Runnegar and Falconer (1981) noted a similarity in both structure and gross pathological effects of Microcystis toxin and phalloidin, a bicyclic peptide from the poisonous mushroom Amanita phalloides. Phalloidin produces a hepatic hemorrhagic necrosis similar to that produced by Microcystis, but they do not extend to the cellular level. Phalloidin produces its toxic effects by inhibiting the depolymerization of F actin to actin; Microcystis does not. The molecular mechanism of action of Microcystis toxin is, at this time, unknown.

Time course studies of dosed rats and mice have been done by several researchers (Slatkin et al., 1983; Ostensvik et al.,

1981; Runnegar and Falconer, 1981). Liver damage can be noted in histological sections as early as 15 min after dosing, with the centrilobular hemorrhagic necrosis steadily progressing outward with time. Platelet counts taken during the course of the toxic reaction show a steady decrease in the circulating platelet count that is inversely proportional to the increase in liver weight (Slatkin et al., 1983; Jones, 1984).

Runnegar and Falconer (1981) noted changes in aspartate amino transferase (ASAT) and lactic dehydrogenase (LDH) at 15 min., and by 30 min had increased to 50 times control levels. Ostensvik et al. (1981) found changes in ASAT commencing at 30 min, but no changes in alanine amino transferase (ALAT) nor bilirubin during the course of toxicity.

Ostensvik et al. (1981) monitored the effect of an extract of a predominately Microcystis water bloom on blood pressure of rats. The blood pressure decreased markedly immediately after intravenous administration, but began increasing at 1.5 min and was back to normal levels within 10 min of injection. During the next 40 min, the blood pressure decreased slowly to very low levels consistent with hemorrhagic shock and remained constant at these low levels until the rats died at about 90 min. Theiss (1984), using purified toxic peptide of M. aeruginosa strain 7820, also found that blood pressure responses, both arterial and venous, were indicative of a direct hepatotoxin which caused death by hemorrhagic shock.

Jackson et al. (1984) inoculated sheep intraruminally with M. aeruginosa water bloom suspension. The majority of the lethally poisoned sheep died within 18 to 23 hr. The carcasses of the sheep showed that the primary site of toxicity was the liver, which had centrilobular to near massive hepatocyte necrosis. Small hemorrhages were also noted in many other areas of the body. Lungs were mildly edematous, but other organs were normal except for the hemorrhages. It was noted that neutrophils were attracted into the lumen of bile ductules without a cholangitis. This has been noted in other animals poisoned with Microcystis and may have diagnostic significance in differentiating algal poisoning from other plant hepatotoxicities. These sheep experiments revealed a sharp dose-response curve in that up to 90% of the lethal dose of bloom could be ingested in a single administration without measurable effect. From the animal response it was estimated that the 27-kg sheep used would have to ingest about 650 cm<sup>3</sup> of the thick algal bloom as it occurred on the pond to provide approximately 30 g of dried cells necessary to cause acute lethal toxicity.

Foxall and Sasner (1981) reported that cladocerans, amphibians, crustacea, and teleosts were not affected by the toxin, but all mammals and birds tested to date are sensitive to it. Some effects have been reported on cardiac muscle and blood

hemostasis (Kirpenko and Kirpenko, 1980). No effect has been reported on skeletal muscle or isolated nerve preparations.

Foxall and Sasner (1981) reported that young hepatocytes in vitro are not affected and neither are young mice nor rats. These mice were not affected by lethal doses of the toxin until they had reached the age of approximately 20 days. This would suggest a possible activation of the toxin by the liver enzyme systems, but to date no one has investigated this possibility. Female mice were slightly more sensitive to the toxin than male mice.

Foxall and Sasner (1981) reported no antibiotic activity against green algae, yeast, or bacteria and no toxicity to certain zooplankton, crayfish, amphibians, and teleosts. However, Grigor'yeva et al. (1977) reported a wide spectrum of antimicrobial activity against Escherichia coli, Shigella flexneri, Salmonella typhimurium, Staphylococcus aureus, Enterococcus, and Candida. The mechanism involved was a decreased thiamine content and inhibition of dehydrogenase activity. Kirpenko et al. (1982) found that Microcystis toxin extracts inhibited the development of saprophytic microflora in artificial ponds.

Kirpenko et al. (1981) found that toxic Microcystis caused embryolethal, teratogenic, and gonadotoxic effects in the rat. Mutagenesis involving anomalies of chromosome and chromatid apparatus was also reported. However, Runnegar and Falconer (1982) reported no mutagenicity when a purified extract was tested by the Ames Salmonella assay.

Reports on Microcystis poisoning are often conflicting. The only consistent pathological findings in Microcystis toxicity are the swollen, blood-engorged liver with hemorrhagic necrosis and the mildly edematous lungs. These conflicting reports may be attributed to possible differences in the effects of different strains of toxic Microcystis, the use of extracts from mixed blooms that contain other algal or bacterial species, or differences in research technique.

Microcystis has been linked to an outbreak in 1979 of hepatoenteritis among an aboriginal population on Palm Island (Queensland, Australia) (Bourke et al., 1983). An epidemiological investigation revealed that 139 children and 10 adults were affected shortly after a copper sulfate treatment of a dense algal bloom in Solomon Dam, the source of the island's reticulated water supply. All of the affected individuals were using water from Solomon Dam and no incidence of disease was reported among persons not receiving water from this supply. The disease had three well-defined phases which were present in the following order: hepatitis phase - 2 days; lethargic phase with severe electrolyte derangement - 1 to 2 days; diarrheal phase - 5

days. Repeated sanitary assessments of the environment of Palm Island failed to uncover any other possible cause for the outbreak.

Falconer et al. (1983a) examined the results of routine assays for hepatic enzymes in plasma of persons who obtained drinking water from a reservoir (Malpas Dam, Armidale, New England, Australia) containing a heavy bloom of toxic *M. aeruginosa* during periods before, during and after the algal bloom. These results were compared with corresponding assays from an adjacent population which did not use water from this source. The residents supplied with water from the bloom-infested Malpas Dam reservoir showed a significant rise in  $\gamma$ -glutamyltransferase (GGT) during the bloom period, while no such increase occurred in residents not receiving their water from the Malpas Dam. ALAT also showed an increase during this period, but it was not statistically significant ( $p < 0.10$ ). ASAT and alkaline phosphatase showed no significant increases. GGT is characteristically released after alcohol or toxin damage to liver cell membranes and is a more sensitive indicator of liver damage than alkaline phosphatase or ASAT.

The toxin of *M. aeruginosa* is normally contained within the algal cells and is released only when the cell is damaged, either by poisoning the algae with copper sulfate, by mechanical rupture of the cell, by breakdown in the stomach, or age-related death of the cell. Damage could occur to the cell when algae in a reservoir are transported through a municipal water system; therefore presence of the blooms in a water supply reservoir might be expected to result in the presence of the toxin in drinking water. Some toxin odor and flavor organics associated with algal blooms can be removed by filtration of reservoir water through sand topped by a layer of granular activated carbon (Falconer et al., 1983b).

The exact composition and structure of *Microcystis* toxins have remained elusive, despite the efforts of many scientists. Louw (1950) working in South Africa was the first to attempt identification of the toxic principle from a *Microcystis* bloom. It is known that variation exists in the toxins from different strains of the algae. The toxin also appears to have an unusual structure which does not lend itself to classical techniques. Working with different strains most workers now agree that the toxins are heptapeptides, with some common and some variable amino acids. On hydrolysis the toxins are found to contain five amino acids and methylamine in approximately equimolar amounts. Amino acids isolated from all various toxin preparations invariably include aspartic acid (or  $\beta$ -methylaspartic acid), glutamic acid, and alanine. Glutamic acid and alanine, the invariant amino acids of Runnegar and Falconer (1981) and Elleman et al. (1978), and methionine, have no free carboxyl groups while the  $\alpha$ -carboxyls of glutamic and  $\beta$ -methylaspartic acid were shown

to be free. The toxin does not react with ninhydrin, nor were any amino groups dansylated in the intact toxin, indicating the absence of any free amino groups (Bishop et al., 1959; Murthy and Capindale, 1970; Rabin and Darbre, 1975; Toerien et al., 1976; Elleman et al., 1978; Botes et al., 1982a; Eloff et al., 1982; Santikarn et al., 1983).

From amino acid analysis, a minimum molecular weight of 654 was derived by Runnegar and Falconer (1981). Other proposed molecular weights, derived by different researchers from various strains and utilizing different techniques, have also been reported (Runnegar and Falconer, 1981). The existence of the toxin as dimers, trimers, or even larger groups of identical or similar subunits could explain the wide variability of proposed molecular weights of the toxin (Eloff et al., 1982). The lack of free amino groups has led to the speculation that the toxin is cyclic (Bishop et al., 1959; Santikarn et al., 1983; Williams, 1983; Botes et al., 1984) and/or has a blocking group on the terminal amide group (Botes et al., 1982a,b).

The extreme hydrophobicity of Microcystis toxins, as exemplified by their chromatographic behavior on paper, cannot be accounted for in terms of their peptide composition and could logically reside in the properties of such a blocking group. The UV spectrum of the toxin shows an absorption maximum at 240 nm; again, this cannot be accounted for by the peptide portion of the molecule, since Botes et al. (1982a) found no aromatic amino acid present. They do suggest that this absorbance peak could be due to the presence of a conjugated diene chromophore in the blocking group.

Eloff et al. (1982) found that the toxins present in different strains were very similar, but variations in toxin composition were found. In some organisms, as many as six different toxins were obtained, but generally one or two toxins accounted for > 90% of the toxin in a single isolate. In all cases the toxin contained  $\beta$ -methylaspartic acid, glutamic acid, alanine, methylamine, and two other amino acids in equimolar ratios. The additional amino acids present in major toxins were as follows: leucine and agrinine, leucine and alanine, tryosine and arginine, methionine and arginine, leucine and tyrosine, alanine and tyosine, or arginine and arginine. The peptide containing leucine and arginine was present in 9 of the 13 toxic isolates.

Botes et al. (1982a) isolated four toxin variants, (BE-2 to BE-5), from a laboratory strain of Microcystis aeruginosa (WR-70). The strain was cultured in a modified Volk and Phinney medium (1968) with the trace element mix of Stainer et al. - (1971). All variants were composed of five amino acid residues with one residue each of  $\beta$ -methylaspartic acid, glutamic acid, and alanine common to all. For the remaining two residues

combinations of leucine and arginine, tryosine and arginine, leucine and alanine, and tyrosine and alanine were found. Methylamine was detected in acid hydrolysates in all cases. Configuration assignments (Botes et al., 1982b) of the  $\alpha$ -carbon atom of the amino acid residues have been made by stereospecific enzymatic transformations, showing that the constant residues are in the D-form, whereas the L-configuration could be assigned to all the variant residues. The relative configuration of the  $\alpha$  carbon atom of  $\beta$ -methylasspartic acid could be made by comparison of the electrophoretic mobility of the toxin-derived residue with literature values for the authentic compound. The presence of N-methyldehydroalanine, which gives rise to methylamine upon acid hydrolysis, has been confirmed by identifying N-methylalanine in the acid hydrolysates of the toxin variants, after reduction of the toxin with sodium borohydride. The use of 400 MHz proton NMR spectroscopy showed the toxins to be more complex than suggested by amino acid analysis alone. Apart from the unambiguous assignment of the amino acid residues, an apolar side chain of 20 carbon atoms was demonstrated. The toxin is thought to exist in dimers, trimers, and other larger aggregates with each subunit consisting of a pentapeptide plus an apolar side chain. In this study the molecular weight of each subunit was estimated at 909.

Based on mass spectrometry, using the BE-4 toxin, Santikarn et al. (1983) and Williams (1983) proposed that the blocking group is a highly unsaturated hydrocarbon with a molecular weight of approximately 313. This would be in addition to the 909-Da subunit of BE-4 toxin. The structure of this side group as reported to be a novel  $\beta$ -amino acid.

There is some new evidence that the peptide may be cyclic. This would explain the resistance of the intact toxin to Edman degradation and degradation by proteolytic enzymes. Neither fast atom bombardment mass spectrometry nor the electron impact mass spectrum of methylated *Microcystis* toxin BE-4 shows evidence of the sequence of ions normally observed in the spectra of linear peptides (Santikarn et al., 1983; Williams, 1983). Botes et al. (1983) showed that the  $\beta$ -amino acid residue was a part of the linear amino acid sequence. The molecular weight of the entire toxin was concluded to be 909. They also proposed the term cyanoginosin for the monocyclic heptapeptides. To designate the variable L-amino acids they propose a two-letter suffix after cyanoginosin. Thus the BE-4 toxin becomes cyanoginosin-IA (Fig. 1).

A low-toxicity strain of *M. aeruginosa* collected by Watanabe and Oishi (1980, 1982) and Watanabe et al (1981) from Lake Suwa in Japan, in contrast to reports of toxins obtained from other world-wide sources, was ninhydrin positive, inactivated by proteases, and not toxic when administered orally to mice. The main amino acids composing this toxin were glutamic acid, aspartic acid, alanine, glycine, arginine, and leucine. The

molecular weight was reported to be approximately 2950, with a minimum of 770, as determined by HPLC. There have been no reported livestock deaths from Microcystis in Japan.

Siegelman et al (1984) presented a method for microdetection of Microcystis toxins. This involves extraction of 20 mg of lyophilized cells with 1 cm<sup>3</sup> of 38% ethanol, 5% n-butanol, 50 mM ammonium acetate for 1 hr. followed by centrifugation for 5 min at 12,000 g. One cm<sup>3</sup> of n-butanol followed by 1 cm<sup>3</sup> of water are added, with vortexing, to the supernatant. The sample is then centrifuged (10 min at 500 g) and the upper phase of n-butanol is collected and dried by evaporation. The residue is extracted with 1 cm<sup>3</sup> of 26% acetonitrile, 500 mM ammonium acetate, stored at 10 degrees centigrade for 16 hr and then centrifuged for 10 min at 12,000 g. These partially purified extracts are stable for several months when stored at -10 degrees centigrade.

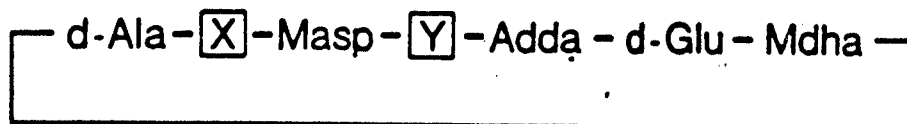


Fig. 1. Proposed general structure for cyclic heptapeptide toxins of Microcystis aeruginosa (Botes et al., 1984). X and Y, variable L-amino acids which can differ between strains. Masp,  $\beta$  methylaspartic acid; Adda, -amino acid residue of 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid; Mdha, methyldehydroalanine.

Aliquots of 5 to 25 mm<sup>3</sup> of these extracts are examined by HPLC as follows:

Precolumn-pellicular octadecyl(C-18) beads (SynChropak, RSC) (0.4 x 5.0 cm)  
Column - 3 m-octadecyl (C-18) silica (Hypersil, Shandon) (0.46 x 5.0 cm)  
Solvent - 6% acetonitrile, 500 mM ammonium acetate, pH 6.0  
Flow - 1.0 cm<sup>3</sup> min<sup>-1</sup>  
Detection - 238 nm at 0.04 AUFS (absorbance units full scale)

In our laboratory we are using a modification of this procedure to purify and compare toxic peptides of Microcystis, Anabaena, and Oscillatoria. These modifications employ C-18 semipreparative columns, G-25 Sephadex gel filtration columns and Bond Elut C-18 preparative cartridges (Theiss, 1984; Theiss and Carmichael,



1986; Krishnamurthy et al. 1986). Kirpenko et al. (1976) have reported on the use of an enzyme photometric assay to detect Microcystis toxins. The method uses cholinesterase and acetylcholine in the presence of the toxin to yield a product which can be measured with the color indicator bromothymol blue.

## WORK IN PROGRESS UNDER CONTRACT WORKSCOPE

### A. Culture Methods for Toxin Producing Strains of Freshwater Cyanobacteria

In the 1950-60's culture media for Cyanobacteria were developed which then allowed for the study of toxins under laboratory conditions. At present, toxic strains of Anabaena, Aphanizomenon, Microcystis, Nodularia and Oscillatoria are cultured in various laboratories around the world. Work efforts under this contract are investigating culture conditions for optimum toxin yield of toxic A. flos-aquae, M. aeruginosa and O. agardhi. A standard method for growth of these toxic cyanobacteria now includes:

- 1) Use of BG-11 (defined inorganic medium) with 1/2 x level nitrate
- 2) Use of 15 liter modified Bellco paddle stirred flasks driven by a low speed magnetic stirrer
- 3) Continuous lighting from a 50/50 mix of cool white fluorescent lights and Vita Lite (R) grow lights. Light intensity is varied from 20-100 u ein/m<sup>2</sup>/sec photosynthetically active irradiation over the growth cycle of the culture (10-15 da).
- 4) Cultures are harvested (30%) every 3-4 days after they reach a stationary phase of growth (60-80 mg/m<sup>3</sup> chlorophyll-a).
- 5) Cells are harvested using a tangential flow Millipore-Pellicon cell concentrator. Cells are lyophilized and stored at -80 degrees C until used for toxin extraction and purification.

Using these methods culture yields are now about 4-500 mg dried cells/liter of culture. Toxin yield is 2-3 mg/g of cells for peptides and 1-2 mg/g of cells for alkaloid neurotoxins.

### B. Extraction, Purification and Analysis of Neurotoxins and Hepatotoxins

A method has been developed which is yielding mg quantities of purified peptide toxins from strains of Anabaena flos-aquae and Microcystis aeruginosa. This method is summarized in Appendix A of this report. Using supplemental funds to this contract issued in the third quarter of the contract equipment is on order and in place to increase the amount of toxin purified per week to about 100 mg. This toxin is to be used in collaborative projects on toxin kinetics and detection at the University of Illinois Veterinary School and USAMRIID - Pathophysiology Division. This is being accomplished through the scale up of

culture facilities, cell concentration/lyophilization, gel filtration and high performance liquid chromatography. Structure work is now available for 3 peptide toxins (2) of Microcystis and 1 of Anabaena). See Appendix A. New laboratory strains and water bloom collections of Microcystis and Oscillatoria are also being purified and analyzed.

Neurotoxins of Aphanizomenon flos-aquae strain NH<sup>-5</sup> resembling neosaxitoxin and saxitoxin (paralytic shellfish toxins) have been isolated and purified (Mahmood and Carmichael, 1986a). A neurotoxin produced by Anabaena flos-aquae strain NRC-525-17 has been isolated and purified. The toxin termed anatoxin-a(s) has the properties of a potent anticholinesterase (Mahmood and Carmichael, 1986b). A summary of its properties include:

- 1) LD<sub>50</sub> intraperitoneal mouse about 50 ug/kg
- 2) Readily absorbed by the oral route
- 3) Mode of death due to antx-a(s) poisoning is overstimulation of cholinergic system
- 4) On isolated muscle preparations antx-a(s) augments the acetylcholine response
- 5) In vitro cholinesterase inhibition studies show antx-a(s) is an irreversible inhibitor.

A continuation of the study on antx-a(s) forms part of this current contract. This work is done as part of a Ph.D. degree in the P.I.'s laboratory and will study the following:

- 1) Central vs. peripheral effects of antx-a(s); its ability to penetrate the blood-brain barrier.
- 2) Effects on nerve/muscle preparation including: miniature end plate potentials.  $\Delta$ PH, Mg and Ca levels.
- 3) Effects on muscle action potentials
- 4) Effects on muscle twitch response
- 5) Effects on release of Ach including binding sites acetylcholinesterase.

Further studies are in progress studying the cellular effects of the peptide hepatotoxins. A electron microscopist Ph.D. level person (Dr. Arun Dabholkar, see attached C.V., Appendix C) has been added to the research staff. The cellular effects of the cyclic peptide (M.W. 994) produced by M. aeruginosa strain 7820 form the bulk of this investigation. Time course events at the cellular level in the liver are being investigated. These time course experiments (0,10,20,30,40,60 min) are being done for acute lethal toxin levels as well as subacute nonlethal levels. Also being investigated are the effects of lethal and nonlethal toxin levels on the isolated perfused rat liver. These studies are designed to clarify and define the toxins mode of action and site of cellular injury using the liver as a primary model system (Appendix B).

C. Collaborative Studies on New Toxic Cyanobacteria Blooms;  
Culture of Toxin Strains and Isolation of Toxins

The following is a summary of these studies carried out during the current contract year.

- 1) Isolation, purification and characterization of peptide toxins produced by Norwegian strains of M. aeruginosa and O. agardhii. This work is in collaboration with The Norwegian Water Research Institute and The Norwegian School of Veterinary Medicine. Results to date have shown that peptides of O. agardhii are different from that of Microcystis toxic strains, found in other parts of the world. It has also shown that certain Oscillatoria strains can produce a toxin with neuromuscular effects. These toxins will be further studied in the coming contract year.
- 2) Isolation and culture of new neurotoxic strains of A. flos-aquae from South Dakota. These strains were obtained from a toxic bloom collected in July of 1985. Losses of animals were reported and signs of toxicity are like that of anatoxin-a(s).
- 3) Isolation and culture of new toxic strains of M. aeruginosa from two locations in Wisconsin. Livestock and dog losses were reported. Signs of toxicity indicate the presence of peptide toxins. Bloom concentrations were extremely heavy, 200-300 g dried cells per liter. At a toxin level of 2-3 mg per gram of dried cells, this means that several kg of pure toxin was present in the bloom material (about 1/2 acre pond).

# LITERATURE CITED

- Adelman, W.J., Jr., Fohlmeister, J.F., Sasner, J.J., Jr. and Ikawa, M. (1982). *Toxicon* 20, 513-516.
- Alam, M., and Euler, K.L. (1981) In "The Water Environment: Algal Toxins and Health" (W.W. Carmichael, ed.), pp. 405-414. Plenum, New York.
- Alam, M. Ikawa, M., Sasner, J.J., Jr., and Sawyer, P.J. (1973). *Toxicon* 11, 65-72.
- Astrachan, N.B., and Archer, B.G. (1981). In "The Water Environment: Algal Toxins and Health" (W.W. Carmichael, ed.), pp. 437-446. Plenum, New York.
- Bates, H.A., and Rapoport, H. (1979). *J. Am. Chem. Soc.* 101, 1259-1265.
- Bishop, C.T., Anet, E.F.L.J., and Gorham, P.R. (1959). *Can. J. Biochem. Physiol.* 37, 453-471.
- Botes, D.P., Kruger, H., and Vilijoen, C.C. (1982a). *Toxicon* 20, 945-954.
- Botes, D.P., Vilijoen, C.C., Kruger, H. Wessels, P.L., and Williams, D.H. (1982b). *Toxicon* 20, 1037-1042.
- Botes, D.P., Tuinman, A.A., Wessels, P.L., Vilijoen, C.C., Kruger, H. Williams, D.H. Santikarn, S., Smith, R.J., and Hammond, S.J. (1984). *J. Chem. Soc. Perkin Trans. I* 2311-2318.
- Bourke, A.T.C., Hawes, R.B., Neilson, A., and Stallman, N.D. (1983). *Toxicon Suppl.* 3, 45.
- Campbell, H.F., Edwards, O.E., and Kolt, R.J. (1977) *Can. J. Chem* 55, 1372-1379.
- Campbell, H.F., Edwards, O.E., Elder, J.W., and Kolt, R.J. (1979). *Pol. J. Chem.* 53, 27-36.
- Carmichael, W.W. (1981). In "The Water Environment: Algal Toxins and Health" (W.W. Carmichael, ed.), pp. 1-12 Plenum, New York.
- Carmichael, W.W. (1982). *S. Afr. J. Sci.* 78, 367-372.
- Carmichael, W.W., and Bent, P.E. (1981). *Appl. Environ. Microbiol.* 41, 1383-1388.

- Carmichael, W.W., and Mahmood, N.A. (1984). In "Seafood Toxins" (E.P. Ragelis, ed.). pp. 377-389. American Chemical Society, Washington, D.C.
- Carmichael, W.W., Biggs, D.F., and Gorham, P.R. (1975). *Science* 187, 542-544.
- Carmichael, W.W., Gorham, P.R., and Biggs, D.F. (1977). *Can. Vet. J.* 18, 71-75.
- Carmichael, W.W., Biggs, D.F., and Peterson, M.A. (1979). *Toxicon* 17, 229-236.
- Carmichael, W.W., Jones, C.L.A., Mahmood, N.A. and Theiss, W.C. (1985). In "Critical Reviews in Environmental Control" (C.P. Straub, ed.) pp 275-313. Chemical Rubber Company Press, Florida.
- Devlin, J.P., Edwards, O.E. Gorham, P.R., Hunter, N.R., Pike, R.K. and Stavric, B. (1977). *Can. J. Chem.* 55, 1367-1371.
- Elleman, T.C., Falconer, I.R., Jackson, A.R.B., and Runnegar, M.T. (1978). *Aust. J. Biol. Sci.* 31, 209-218.
- Eloff, J.N., Siegelmann, H.W., and Kycia, H. (1982). *S. Afr. J. Sci.* 78, 377 (Abstract).
- Falconer, I.R., Jackson, A.R.B., Langley, J. and Runnegar, M.T. (1981) *Aust. J. Bio. Sci.* 34, 179-187.
- Falconer, I.R., Beresford, A..M., and Runnegar, M.T.C. (1983a) *Med. J. Aust.* 1, 511-514.
- Falconer, I.R., Runnegar, M.T.C., and Huynh, V.L. (1983b) Tenth Fed. Convention Aust. Water Wastewater Assoc., 10th Sydney, April 26-1.
- Farber, J.L. (1981). *Life Sci.* 9, 1289.
- Foxall, T.L., and Sasner, J.J., Jr. (1981). In "The Water Environment: Algal Toxins and Health" (W.W. Carmichael, ed.). pp 365-387. Plenum, New York.
- Grabow, W.O.K., DuRandt, W.C., Prozesky, O.W., and Scott, W.E. (1982). *Appl. Envirn. Microbiol.* 43, 1425-1433.
- Grigor'yeva, L.V., Kirpenko, Yu A., Oriovskiy, V.M. and Stankevich, V.V. (1977). *Gidrobiol. Zh.* 13, 57-62 (Ukranian).
- Huber, C.S. (1972). *Acta Crystallogr. vol. B.* 28, 2577-2582.

- Ikawa, M., Wegener, K., Foxall, T.L., and Sasner, J.J., Jr.  
(1982) *Toxicon* 20, 747-752.
- Jackim, E., And Gentile, J.H. (1968). *Science* 162, 915-916.
- Jackson, A.R.B., McInnes, A. Falconer, I.R., and Runnegar, M.T.C.  
(1984). *Vet. Pathol.* 21, 102-113.
- Jones, C.L.A. (1984) MSc. thesis, Wright State University,  
Dayton, Ohio
- Kirpenko, Yu. A., Lukina, L.F., Orlovskiy, M., and Peskov, V.A.  
(1976). *Bull. Izobretenia* 16, 125-126 (Ukranian).
- Kirpenko, Yu A., Sirenko, L.A., and Kirpenko, N.I. (1981).  
In "The Water Environment: Algal Toxins and Health"  
(W.W. Carmichael, ed.) pp.257-269. Plenum, New York.
- Kirpenko, Yu A., Stankevich, V.V., and Kirpenko, N.I. (1982)  
*Gidrobiol. Zh.* 18, 34-36 (Ukranian).
- Krishnamurthy, T., Carmichael, W.W. and Sarver, E.W. (1986).  
*Toxicon* 24, 865-873.
- Louw, P.G.J. (1950), *S. Afr. Ind. Chem.* 4, 62-66.
- Mahmood, N.A., and Carmichael, W.W. (1986a). *Toxicon.* 24, 175-  
186.
- Mahmood, N.A. and Carmichael, W.W. (1986b). *Toxicon.* 24,  
425-434.
- Murthy, J.R., and Capindale, J.B. (1970). *Can. J. Biochem.* 48,  
508-510.
- Ostensvik, O., Skulberg, O.M., and Solim, N.E. (1981). In  
"The Water Environment: Algal Toxins and Health"  
(W.W. Carmichael, ed.), pp. 315-324. Plenum, New York.
- Rabin, R., and Darbre, A. (1975) *Biochem. Soc. Trans.* 3,  
428-430.
- Runnegar, M.T.C. and Falconer, I.R. (1981) In "The Water  
Environment: Algal Toxins and Health" (W.W. Carmichael,  
ed.), pp. 325-342. Plenum, New York.
- Runnegar, M.T.C., and Falconer, I.R. (1982). *S. Afr. J. Sci.* 78.
- Santikarn, S., Williams, D.H., Smith, R.J. Hammond, S.J.,  
Botes, D.P., Tuinman, A., Wessels, P.L., Viljoen, C.C.  
and Kruger, H. (1983). *J. Chem. Soc. Chem. Commun.* 12,  
652-654.

- Sasner, J.J., Jr., Ikawa, M., Foxall, T.L., and Watson, W.H. (1981). In "The Water Environment: Algal Toxins and Health" (W.W. Carmichael, ed.) pp. 389-402.
- Sasner, J.J., Jr., Ikawa, M., and Foxall, T.L. (1984). In "Seafood Toxins" (E.P. Ragelis, ed.), pp 391-406, American Chemical Society, Washington, D.C.
- Sawyer, P.J., Gentile, J.H., Sasner, J.J., Jr (1968). *Can. J. Microbiol.* 14, 1199-1204.
- Siegelman, H.W., Adams, W.H., Stoner, R.H., and Slatkin, D.N. (1984). In "Seafood Toxins" (E.P. Ragelis, ed.), pp. 407-413. American Chemical Society, Washington, D.C.
- Skulberg, O.M., Codd, G.A., and Carmichael, W.W. (1984) *Ambio* 13, 244-247.
- Slatkin, D.N., Stoner, R.D., Adams, W.H., Kycia, J.H. and Siegelman, H.W. (1983) *Science* 220, 1383-1385.
- Smith, M.T., Thor, H., and Orrenius, S. (1981) *Science* 213, 1257.
- Spivak, C.E., Witkop, B., and Albuquerque, E.X. (1980). *Mol. Pharmacol.* 18, 384-394.
- Stainer, R.Y., Kunisaa, R., Mandel, M., and Cohn-Bazire, (1971) *Bacteriol. Rev.* 35, 171-205.
- Theiss, W., and Carmichael, W.W. (1986). In "Mycotoxins and Phycotoxins" (P.S. Steyn and R. Vleggaar, Eds.) pp. 353-364 Elsevier Sci. Publishers, Amsterdam.
- Toerien, D.F., Scott, W.E., and Pitout, M.J. (1976). *Water S. Afr. (Pretoria)* 2, 160-162.
- Volk, S.L., and Phinney, H.K. (1968) *Can. J. Bot.* 46, 619-630.
- Wantanabe, M.F., and Oishi, S. (1980) *Can. J. Limnol.* 41, 5-9.
- Wantanabe, M.F., and Oishi, S. (1982). *Appl. Environ. Microbiol.* 43, 819-822.
- Wantanabe, M.F., Oishi, S., and Makao, T. (1981). *Verh. Int. Verein. Limnol.* 21, 1441-1443.
- Williams, D.H. (1983). *Int. J. Mass Spectrosc. Ion, Phys.* 53, 37-44.
- Wong, S.H., and Hindin, E. (1982) *Am. Water Works Assoc. J.* 74, 528-529.



## APPENDIXES

**APPENDIX A**

**MANUSCRIPT SUBMITTED FOR REVIEW IN TOXICOM**

**SUPPORTED IN PART BY CONTRACT NO DAMD17-84-C-4156**

Investigations of freshwater cyanobacteria (blue-green algae)  
toxic peptides. I. Isolation, purification and characterization  
of peptides from Microcystis aeruginosa and Anabaena flos-aquae.\*

T. Krishnamurthy<sup>1</sup>, W.W. Carmichael<sup>2</sup>,  
E.W. Sarver<sup>1</sup>

<sup>1</sup>Chemical Research and Development Center  
Aberdeen Proving Ground, Md. 21010

<sup>2</sup>Department of Biological Sciences  
Wright State University  
Dayton, Oh 45435

(Accepted for publication \_\_\_\_\_)

Running title: Peptide Toxins of Microcystis aeruginosa and  
Anabaena flos-aquae

\*Now published - Toxicon. 24(9): 865-873. 1986.

### Abstract

T. Krishnamurthy, W.W. Carmichael and E.W. Sarver. Investigations of freshwater cyanobacteria (blue-green algae) toxic peptides. I. Isolation, purification and characterization of peptides from Microcystis aeruginosa and Anabaena flos-aquae Toxicon 24, No. 9, 1985.

Toxic peptides of freshwater cyanobacteria (blue-green algae) from two European Microcystis aeruginosa and one Canadian Anabaena flos-aquae were purified and examined by amino acid analysis and mass spectrometry. A toxic fraction from a butanol/methanol extract of toxic lyophilized cells was separated by G-25 gel filtration and purified by HPLC using a C-18 semiprep Column. A toxic peak with the same elution time was detected for each of the three toxic cyanobacteria. The desalted purified toxins (LD<sub>50</sub> intraperitoneal mouse, 50 ug/mg) showed signs of poisoning identical with previous literature reports of hepatotoxic peptides from Microcystis. On hydrolysis and amino acid analysis all three toxins showed a similar profile consisting of equimolar amounts of glutamic acid, alanine, arginine and leucine. 8-methyl aspartic acid was identified in all of the toxic peptides. The Fast Atom Bombardment (FAB) mass spectra of the toxins indicated the molecular weight to be 994 for all the peptides. The peptides were proposed to be cyclic based on the absence of sequence ions in their corresponding FAB spectra.

## INTRODUCTION

Heavy growths (water blooms) of cyanobacteria (blue-green algae) are a regular occurrence in eutrophic natural and manmade water bodies during the summer and fall months. In addition to the impaired use of these water bodies for drinking and recreation purposes, several of the more common bloom forming species can produce toxins (1,2,3). These toxins have caused loss of cattle and wild animals, due to the consumption of the contaminated water and the bloom mass, in several countries throughout the world (1-5). Human toxication has also been implicated in several instances (6-7). Increased pollution in urban, recreational, and agricultural water sources seem to contribute to conditions for the growth of toxic and nontoxic blooms. These toxins in municipal and recreational water supplies create special problems for protection of human health and increase the costs for water treatment (8,9). Of about 10 suspected toxic cyanobacteria genera, blooms of Microcystis aeruginosa continue to be the most commonly reported (1).

The toxins isolated from M. aeruginosa produce distinct hemorrhagic necrosis to the liver in test animals (2). These hepatotoxins have been detected from water blooms in Australia, Canada, Great Britain, Japan, Norway, South Africa and the U.S.S.R. (2). They have been reported to be small molecular weight amino acids with a weight in the range of 1000 daltons (2). The available structural information on these toxins is very limited. Hence investigations have been initiated in several countries in order to elucidate their structure, study their properties, and to develop methods of detection and possible decontamination of the affected sources (2,4).

The only known unambiguous structural elucidation of the hepatotoxin from M. aeruginosa is reported by researchers using South African toxic strains (10-13). They have described the isolation, purification and characterization of four peptides from a single laboratory culture isolated from a waterbloom. The amino acid analysis revealed five residues in each. Alanine, glutamic acid and  $\beta$ -methylaspartic acid were detected in all. The two uncommon moieties were found to be combinations of leucine and arginine, tyrosine and arginine, leucine and alanine, and tyrosine and alanine (10). The configuration of the asymmetric carbons in the common and uncommon moieties were determined to be d- and l- forms respectively (11).

Based on fast atom bombardment mass spectroscopy of one of the toxin variants, termed BE-4 (M.W. 909), Santikarn et al. (12) proposed the toxin to be cyclic. This reaffirmed the original observation of Bishop et al. (14) who proposed that the toxin of M. aeruginosa NRC-1 was a cyclic polypeptide. The structure of BE-4 was unambiguously defined based on nuclear magnetic resonance, mass spectral data of the intact peptide and its chemical

degradation product (13). We have initiated investigations in order to elucidate the total structures of several cyanobacterial toxic peptides. It is felt that these studies will lead to methods of detection and analysis in environmental samples plus possible ways to decontaminate affected water supplies. The toxins studied were two laboratory cultures and one natural water bloom. The laboratory cultures included *M. aeruginosa* strain 7820 and *A. flos-aquae* strain S-23-g-1. Strain 7820 was isolated by G.A. Codd from a toxic waterbloom near Dundee, Scotland in 1976 (15). Strain S-23-g-1 was isolated from a lake near Saskatchewan, Canada in 1975 (16). The waterbloom material was collected from Akersvatn (Akers Lake) near Oslo, Norway in August of 1984. This bloom material was provided by O.M. Skulberg of the Norwegian Water Resources Institute. It should be noted that prior to this study *A. flos-aquae* was not known to produce hepatotoxic peptides.

## Materials and Methods

### Toxin Purification

Toxin from all three sources was isolated from lyophilized cells according to a procedure modified from that of Siegelman et al. (17) listed in Fig. 1.

Toxicity Testing. Replicate ICR Swiss male mice (18-24 g) were used to monitor all stages of the purification procedure. Toxin extracts and cells were injected by the intraperitoneal (i.p.) route. Signs of poisoning were monitored and characteristic liver hemorrhage, including the ratio of the liver weight versus the body weight, were noted.

Amino Acid Analysis: Two methods were used for amino acid analysis. In the first method peptides (50-100 nMole) were digested with 6N HCl containing 0.5% phenol and 0.5% mercaptoethanol in a sealed ampoule under vacuum at 112 C for 24 hours. The released amino acids were analyzed using a Liqui-Mat amino acid analyzer. The product was loaded on the standard Mitsubishi exchange resin MCI-gel CK 08F column (4 mm x 16 cm) and eluted with sodium citrate buffer (0.18-0.20 M). Ninhydrin (0.1 M) in DMSO was used for the post-column derivatization in order to detect the amino acids at 440 nm and 570 nm. The elution times were calibrated using  $\alpha$ -amino,  $\beta$ -guanidinopropionic acid hydrochloride. In the second method peptides (5 ug) were hydrolyzed in 6N HCl at 106 C for 24 hrs. The released amino acids were precolumn derivitized with Phenylisothiocyanate (PITC) and the phenylthiocarbamyl (PTC) amino acids were analyzed using a Waters Pico Tag HPLC system. The derivatives were loaded on a C-18 (15 cm x 4.6 mm) column and eluted using 0.138 M sodium acetate trihydrate in water to 60% acetonitrile in 8 min. The column flow rate is 1.0 mL/min and detection is at 254 nm.

**Mass spectra:** Mass spectra were obtained from a standard Finnigan-Mat TSQ mass spectrometer with the mass range up to 1,700 daltons. The fast atom bombardment (FAB) spectra were recorded by placing the sample (50-100 nMole) in a glycerol matrix with oxalic acid on the copper sample stage and introduced into the source at 60 C. The sample was bombarded with 8 kv krypton atoms maintaining the source temperature at 60 C.

## RESULTS AND DISCUSSION

The toxins were isolated from *Microcystis aeruginosa* 7820, *Anabaena flos-aquae* S-23-g-1, and Akersvatn bloom and purified by the procedure described in Figure 1. The toxin extracts were separated from the plant pigments using Analytichem Bond Elute C-18 column and G-25 gel filtration. Purification of this extract was by high performance liquid chromatography (HPLC) using a semi-prep reverse-phase C-18 column and eluting with 0.01 M ammonium acetate buffer in 26% aqueous acetonitrile. The purified extract was desalted by loading again on the semi-prep column and eluting with 26% aqueous acetonitrile. The process was repeated in order to ensure the total removal of the salts from the toxin, which is very essential for the FAB mass spectral investigation in addition to the accurate determination of purified toxin. The purity of the toxin was further ensured by acid (Sigma). Two peaks with the ratio of 7:1 were observed eluting at 2.2 and 2.7 minutes (Figure 4A). This was probably due to the two possible diastereomers for the  $\beta$ -methyl aspartic acid. The enantiomer of each of these must be co-eluting with the other. One of the diastereomers seemed to co-elute with the glutamic acid, which accounted for the observed higher molar ratio of 1.4 for glutamic acid. This was established by analyzing the spiked amino acid standard mixture (Figure 4 B & C). The toxin from S-23-g-1 was also spiked with  $\beta$ -methyl aspartic standard (results not shown). The resulting profile showed an increase both in the glutamic acid peak (2.2. min) and the peak which corresponded with the 2.7 min peak of the  $\beta$ -methyl aspartic standard. Based on these results, it was concluded that all the investigated peptides contain alanine, arginine, glutamic acid, leucine and  $\beta$ -methylaspartic acid in equi-molar ratio. In addition they all have an unidentified amino acid; the elution time of which (8.5, Figure 3) was observed to be the same in all three toxin hydrolysates.

The Fast Atom Bombardment mass spectra of AKERTOX in glycerol and thioglycerol were recorded and the molecular weight of the peptide was determined to be 994. The former matrix was found to be better suited and the addition of oxalic acid to the glucerol matrix considerably improved the total ion current of the quasi-molecular ion. The FAB spectrum of the peptide in glycerol matrix is shown in Figure 5. The lack of sequence information in the FAB spectra indicates the peptide to be cyclic

(18). The FAB spectrum of 781 toxin in glycerol matrix indicated two quasi-molecular ions  $m/z$  995 and 1013 without any sequence information. These peptides were purified by repeated HPLC separation and the FAB spectrum of the abundant peptide is given in Figure 6. The products from the acid hydrolysis, trypsin cleavage and the Edman Degradation of the 7820 peptide were found to be the same. Only one compound with the molecular weight of 1012 was detected in all the reaction mixtures. It was interpreted that the peptide with the molecular weight 994 ring, opened during the process and formed the product with the addition of a water molecule (see Figure 2a). This product and the other component in the original peptide mixture with the same molecular weight of 1012 did not undergo any further reactions. All these observations were further proof for the assumption that these peptides are similar to the Akertox peptide. The FAB spectrum of the S-23-g-1 peptide indicated the molecule to have a molecule weight of 994. Absence of the sequence information in this case indicated that this peptide is cyclic as well. The other common feature noted in all the peptides was the presence of  $m/z$  135 ion in their FAB spectra. Based on these observations it is proposed that these peptides, which originated from different geographical areas, are cyclic with possible similar amino acid sequences and structure. Further investigations in order to elucidate the total structure of all these peptides are in progress.



#### ACKNOWLEDGEMENTS

The authors would like to thank G.A. Codd and O.M. Skulberg respectively for the culture and lyophilized cells of toxic Microcystis. The technical assistance, in extraction, purification and amino acid analysis of toxins by J. Eschedor is also appreciated. This work was supported in part by a United States Army Medical Research and Development Command contract and an NIH Biomedical Seed Grant to WWC.

## REFERENCES

- W.W. Carmichael, Ed. (1981). The Water Environment: Algal Toxins and Health, Plenum Press; New York, 491 pp.
- W.W. Carmichael, C.L.A. Jones, N.A. Mahmood and W.C. Theiss. (1985). Algal Toxins and Water-Based Diseases. CRC Critical Reviews in Environmental Control. 15, 275.
- W.W. Carmichael and N.A. Mahmood. (1984). Toxins from Fresh water Cyanobacteria. In Seafood Toxins, p. 377 (Ragelis, E., Ed.) American Chemical Society. Symposium Series 262, Washington, D.C.
- O.M. Skulberg, G.A. Codd and W.W. Carmichael. (1984) Toxic Blue-green Algal Blooms in Europe: A Growing Problem. AMBIO, 13, 244.
- V.R. Beasley, R.W. Coppock, J. Simm, R. Ely, W. Buck, R.A. Corley, D.M. Carlson and P.R. Gorham. (1983) Apparent Blue-green Algae Poisoning in Swine Subsequent to Ingestion of a Bloom Dominated by Anabaena spiroides. J. Am. Vet. Med. Assoc. 182, 413.
- W.H. Billings. (1982) Water Associated Human Illness in Northeast Pennsylvania and Its Suspected Association with Blue-green Algae Blooms. In The Water Environment: Algal Toxins and Health, p. 243 (Carmichael, W., Ed.) Plenum Press, New York and London.
- Falconer, I.R., A.M. Beresford and M.T.C. Runnegar. (1983) Evidence of Liver Damage by Toxin from a Bloom of the Blue-green Algae, Microcystis aeruginosa. Med. J. Aust. 1, 511.
- Hoffman, J.R.H. (1976) Removal of Microcystis Toxins in Water Purification Processes. Water South Africa 2, 58.
- Falconer, I.R., Runnegar, M.T.C., and Huynh, V.L. (1983). Effectiveness of Activated Carbon in the Removal of Algal toxin from Potable Water Supplies: A Pilot Plant Investigation. p. 26 In Technical papers presented at the Tenth Federal Convention of the Australian Water and Wastewater Association. Sydney.
- Botes, D.P., Kruger, H. and Viljoen, C.C. (1982) Isolation and Characterization of Four Toxins from the Blue-green Algae, Microcystis aeruginosa. Toxicon 20, 945.
- Botes, D.P., Viljoen, C.C., Kruger, H. Wessels, P.L. and Williams, D.H. (1982). Configuration Assignments of the Amino Acid Residues and the Presence of N-Methyldehydroalanine in Toxins from the Blue-green Algae Microcystis

aeruginosa. Toxicon. 20, 1037.

Santikarn, S., Williams, D.H., Smith, R.J. Hammond, S.J. Botes, D.P., Tuinman, A., Wessels, P.L., Vilgoen, C.C. and Krueger H. (1983). A Partial Structure for the Toxin BE-4 from the Blue-green Algae, Microcystis aeruginosa. J. Chem. Soc. Chem. Commun. 12, 652.

Botes, D.P., A.A. Tuinman, P.L. Wessels, C.C. Viljoen and H. Krueger (1984). The Structure of Cyanoginosin -L.A. a Cyclic Heptapeptide Toxin from the Cyanobacterium Microcystis aeruginosa. J. Chem. Soc. Perkin. Trans. 1, 2311.

Bishop, C.T., E.F.L.J. Anet and P.R. Gorham. (1959) Isolation and Identification of the Fast-death Factor in Microcystis aeruginosa NRC-1. Can. J. Biochem. Physiol. 37, 453.

Codd, G.A. and W.W. Carmichael. (1982) Toxicity of a Clonal Isolate of the Cyanobacterium Microcystis aeruginosa from Great Britain. FEMS Microbiology Letters. 13, 409.

Carmichael, W.W. and P.R. Gorham. (1978) Anatoxins from Clones of Anabaena flos-aquae Isolated from Lakes of Western Canada. Mitt. Internat. Verein. Limnol. 21, 285.

Siegelman, A.W., N.H. Adams, R.D. Stoner and D.W. Slatkin. (1984) Toxins of Microcystis aeruginosa and Their Hematological and Histopathological Effects. In Seafood Toxins, p. 407 (Ragelis, E., Ed.) American Chemical Society Symposium Series 262. Washington, D.C.

Tomer, B., F.W. Crow, M.L. Gross and C.D. Koppler. (1984) Fast Atom Bombardment Combined with Tandem Mass Spectrometry for the Determination of Cyclic Peptides. Anal. Chem. 56, 880.

TABLE 1. AMINO ACID ANALYSIS BY LIQUI-MAT A.A. ANALYZER

Peptide Source	Detected Amounts of amino acids in nMole (Ratio with respect to alanine)				
	Alanine	Arginine	Glutamic Acid	Leucine	Number of Unidenti- fied amino acids
AKERTOX (Akersvatn)	86(1)	80(0.9)	87(1.0)	96(1.1)	2
S-23-G-1	58(1)	52(0.9)	61(1.1)	61(1.1)	2
7820	155(1)	133(0.9)	162(1.0)	160(1.0)	2

TABLE 2. Amino Acid Analysis by Waters Pico Tag® HPLC System

	Retention Time (Minutes)		Detected Amt. in Picosole (Molar ratio based on Alanine)	
Standard	AKERTOX	7820	S-23-g-1	AKERTOX 7820 S-23-g-1
Alanine	6.1	6.1	6.1	66(1.0) 76(1.0) 202(1.0)
Arginine	5.8	5.8	5.8	57(0.9) 64(0.8) 222(1.1)
Glutamic Acid	2.1	2.2	2.2	92(1.4) 103(1.4) 299(1.5)
Leucine	9.9	9.8	9.9	75(1.1) 77(1.0) 223(1.1)
β -Methylaspartic Acid	2.7	2.7	2.7	44(0.7) 40(0.5) 109(0.5)

Fig. 1. EXTRACTION AND PURIFICATION PROCEDURE FOR HEPATOTOXIC PEPTIDES OF MICROCYSTIS AERUGINOSA AND ANABAENA FLOS-AQUAE. Purified toxins have an approximate i.p. LD<sub>50</sub> in mice of 50 ug/kg, with a survival time of 30-90 min. Each gram of lyophilized cells contains between 1-4 mg of toxin. Recovery of the toxin is between 70-75%.

Fig. 2. HIGH PERFORMANCE LIQUID CHROMATOGRAPH OF PURIFIED TOXINS FROM (A) MICROCYSTIS AERUGINOSA STRAIN 7820 (10 UG), (B) AKERSVATN WATER BLOOM (8 UG) AND (C) ANABAENA FLOS-AQUAE S-23-G-1 (8 ug). Column conditions were: column, ALTEX C-18 4.6 mm x 25 cm; solvent, 0.01 M ammonium acetate in 26% acetonitrile/water; flow rate, 1 mL/min; wavelength, 240 nm; AUFS, 0.1.

Fig. 3 HIGH PERFORMANCE LIQUID CHROMATOGRAPH, AMINO ACID PROFILE OF MICROCYSTIS AERUGINOSA AKERTOX (5 UG). Analysis is by Waters Pico Tag precolumn derivitization with phenylisothiocyanate to yield phenylthiocarbamyl amino acids. Pico mole amounts of each amino acid are given in Table 2. M. aeruginosa 7820 and A. flos-aquae S-23-g-1 toxin gave similar profiles. PITC DERIV. = unidentified phenylisothiocyanate derivative produced during sample derivitization. Ordinate units in millivolts.

Fig. 4 A. HIGH PERFORMANCE LIQUID CHROMATOGRAPH OF METHYL ASPARTIC ACID (5 ug), USING WATERS PICO TAG ANALYSIS, SHOWING THE TWO DIASTEREOMERSE WHICH ARE ELUTING AT 2.2 AND 2.7 MIN. GLU = POSITION WHERE GLUTAMIC ACID WOULD ELUTE IF PRESENT.

B. High performance liquid chromatograph profile of Pierce amino acid standards (250 pico moles each) using Waters Pico Tag analysis. Note approximately equal sized peaks for aspartic and glutamic acids.

C. High performance liquid chromatograph profile of Pierce amino acid standards (250 pico moles each) using Waters Pico Tag analysis. Sample is spiked with 5 ug of  $\beta$ -methyl aspartic acid. Note enhanced glutamic acid peak (2.2 min) and peak at 2.7 min.

Fig. 5 FAST ATOM BOMBARDMENT MASS SPECTRUM OF M. AERUGINOSA AKERTOX PEPTIDE IN GLYCEROL MATRIX.

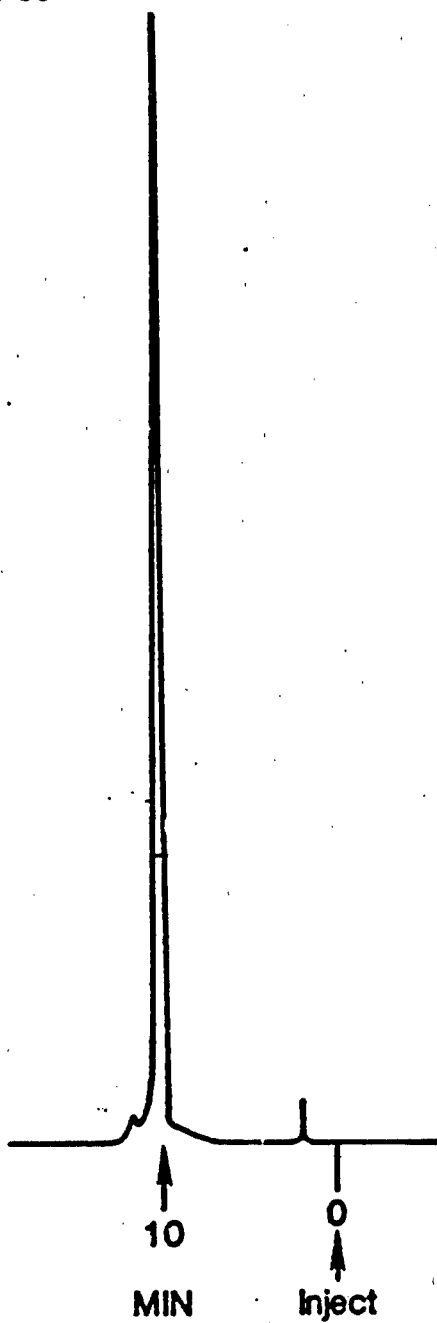
FIG. 6 FAST ATOM BOMBARDMENT MASS SPECTRUM OF M. AERUGINOSA 7820 PEPTIDE IN GLYCEROL MATRIX.

FIG. 1

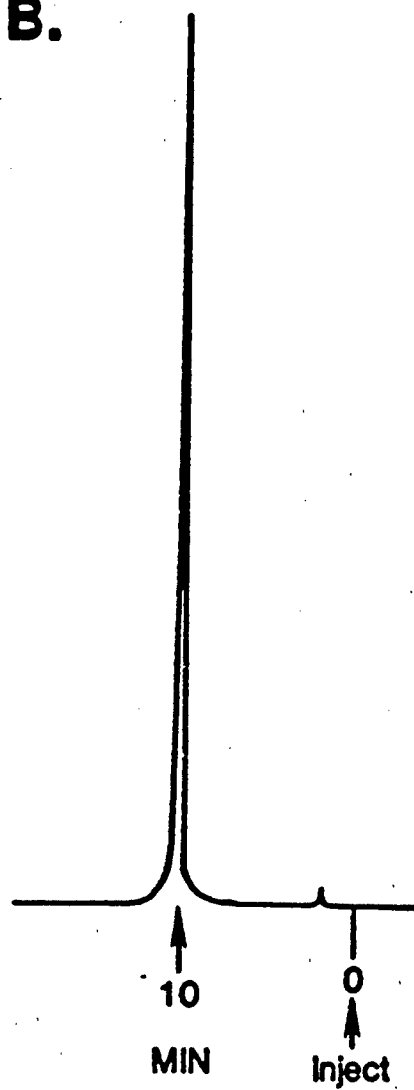
PROCEDURE FOR THE EXTRACTION OF HEPATOTOXIC  
PEPTIDES FROM FRESHWATER CYANOBACTERIA

- 1) 1 GM CELLS + 200 ML 5% BUTANOL-20% METHANOL-75% WATER  
STIR 1-3 HRS AT 4 DEG C.  
CENTRIFUGE 100,000 X G - 1 HR AT 4 DEG C.  
REPEAT 3 TIMES WITH CELL PELLET
- 2) COMBINE SUPERNATANTS  
REDUCE VOLUME TO 300-350 ML BY AIR DRYING
- 3) SUPERNATANT PASSED THROUGH ANALYTICHEM BOND ELUTE C-18 COLUMN  
ELUTE TOXIC FRACTION WITH 3-5 ML 100% MECH  
REPEAT PROCESS 3-4 TIMES (3-4 X)
- 4) DRY COMBINED METHANOL EXTRACT WITH NITROGEN  
DISSOLVE RESIDUE IN 5 ML WATER  
PASS THROUGH 3.0 MICRON MILLIPORE FILTER
- 5) K-26 PHARMACIA COLUMN (26 MM X 80 CM)  
WITH 100 GM SEPHADEX G-25  
ELUTE IN 5% METHANOL WATER  
MONITOR AT 240 NM  
TOXIN IS FIRST LARGE PEAK OFF THE COLUMN
- 6) HPLC-ALTEX C-18 9.4 MM X 25 CM  
0.01 AMMONIUM ACETATE IN 26% ACETONITRILE/WATER  
FLOW RATE 3 ML/MIN  
MONITOR AT 240 NM
- 7) LYOPHILIZE TOXIC PEAK  
DESALT TOXIN BY HPLC  
AS IN STEP 6 - USING 26% ACETONITRILE/WATER
- 8) STORE TOXIN AT -80 DEGREES C UNTIL USE

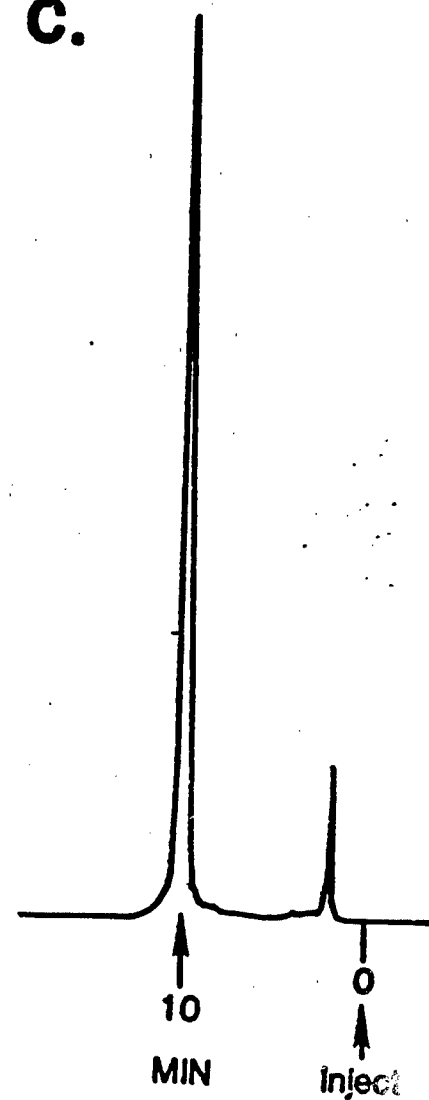
**A.**



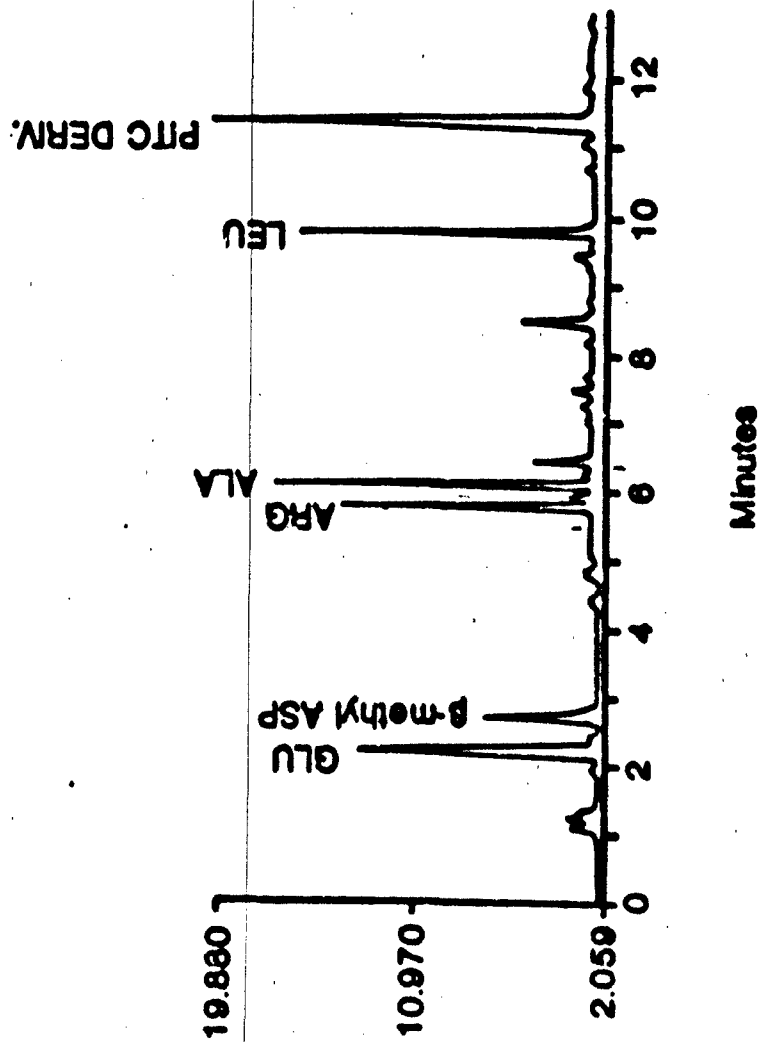
**B.**



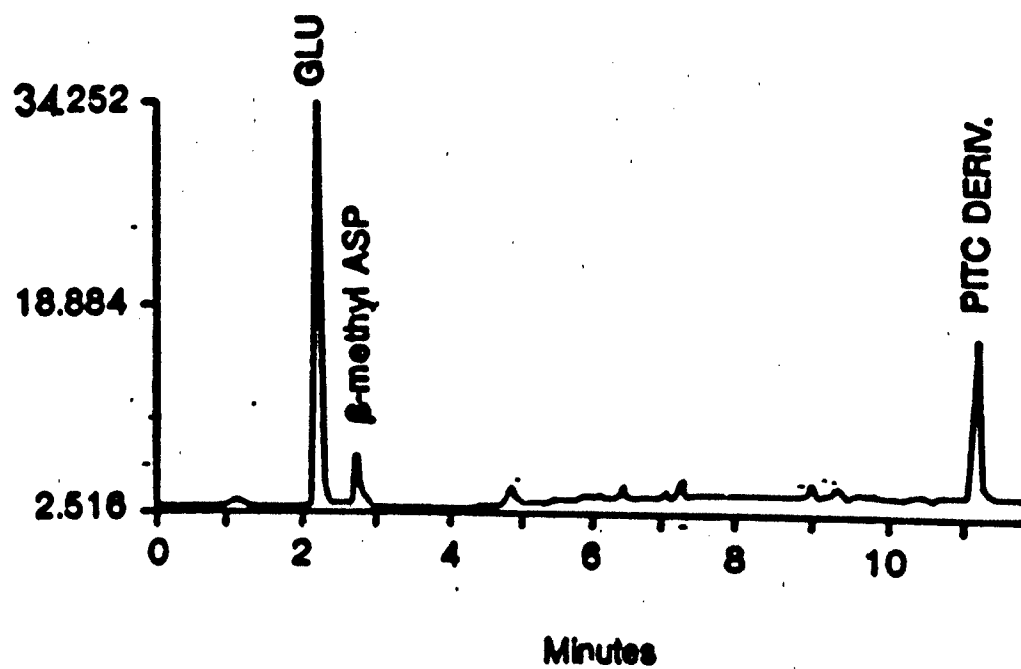
**C.**



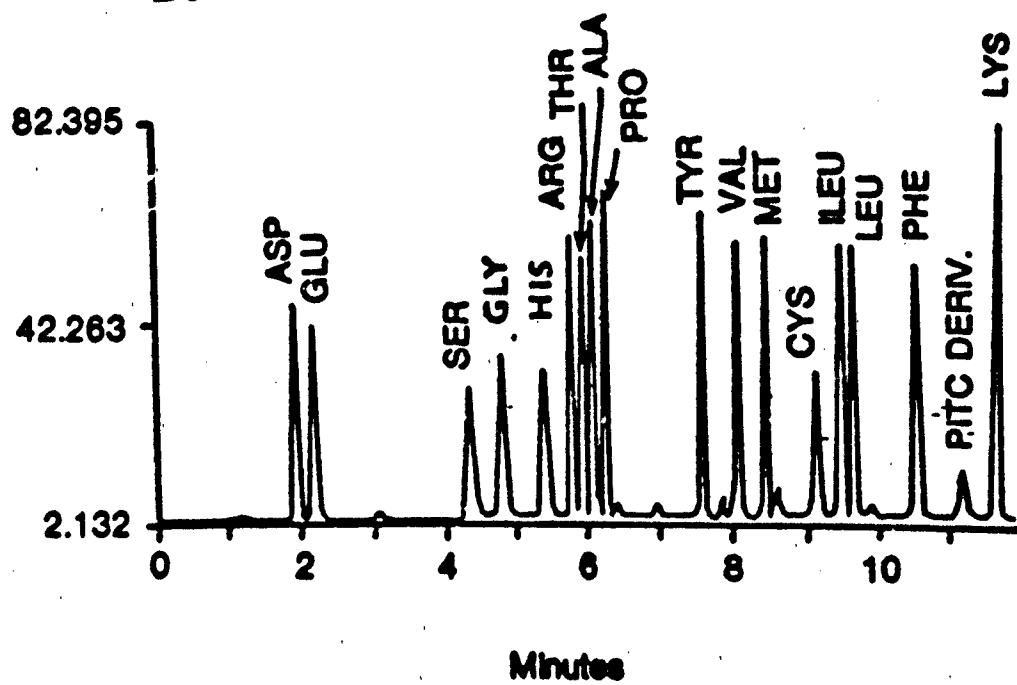




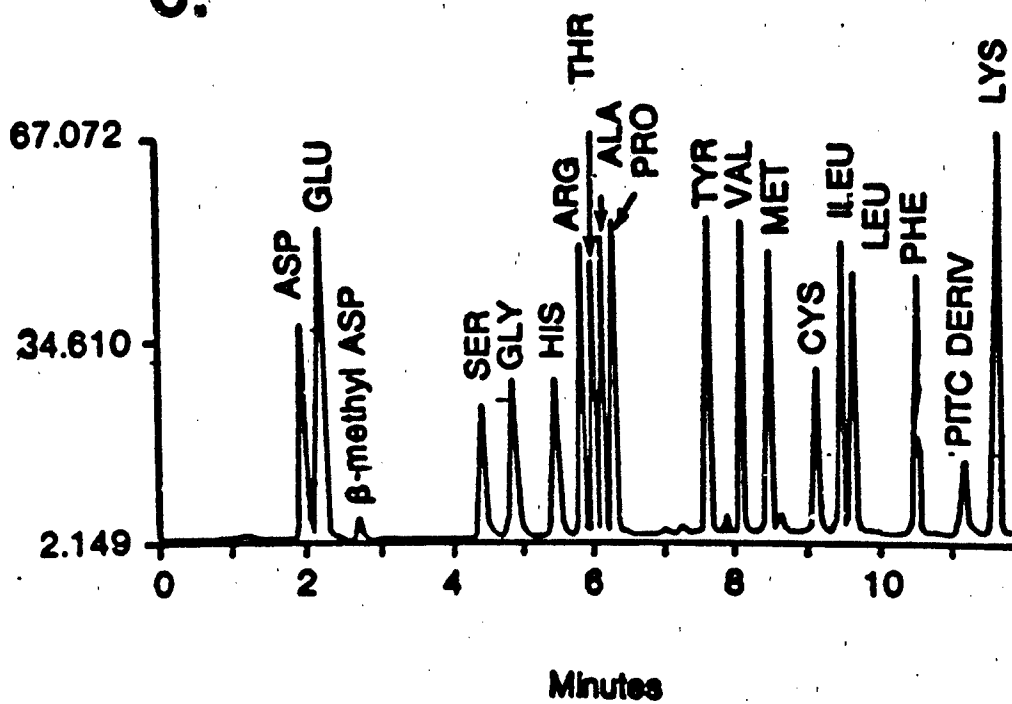
**A.**



B.



C.



# MASS SPECTRUM

12/11/84 14:18:00 + 0:16

SAMPLE: AKERTX

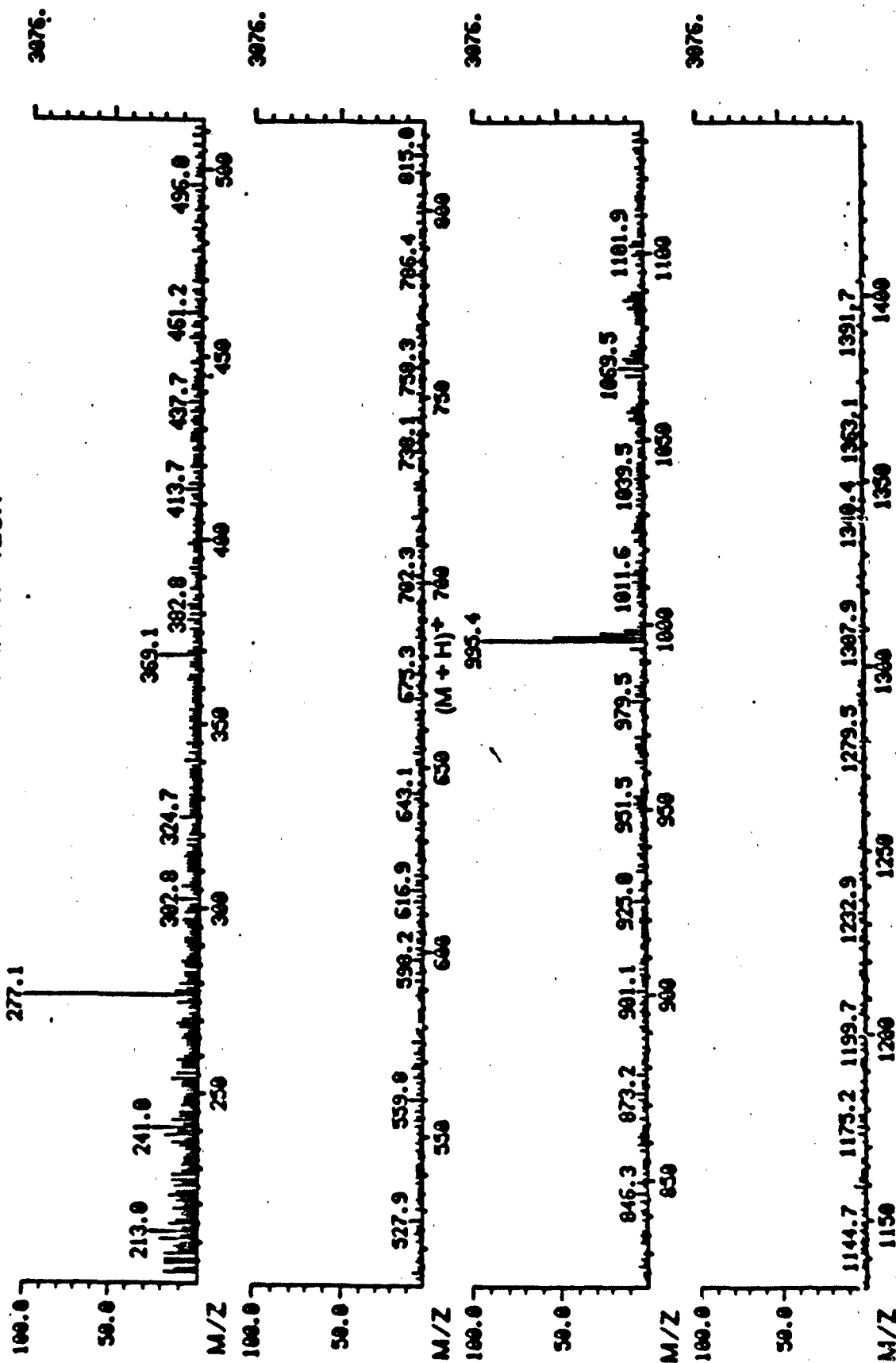
CONDS.: +/CI - KR/Q1 MASS SPECTRUM/7KV 12UA

DATA: HTK4 #12

BASE M/Z: 277

CALI: CAL1031Q1 #1

RIC: 128000



# MASS SPECTRUM

12/11/84 12:41:00 +0:42

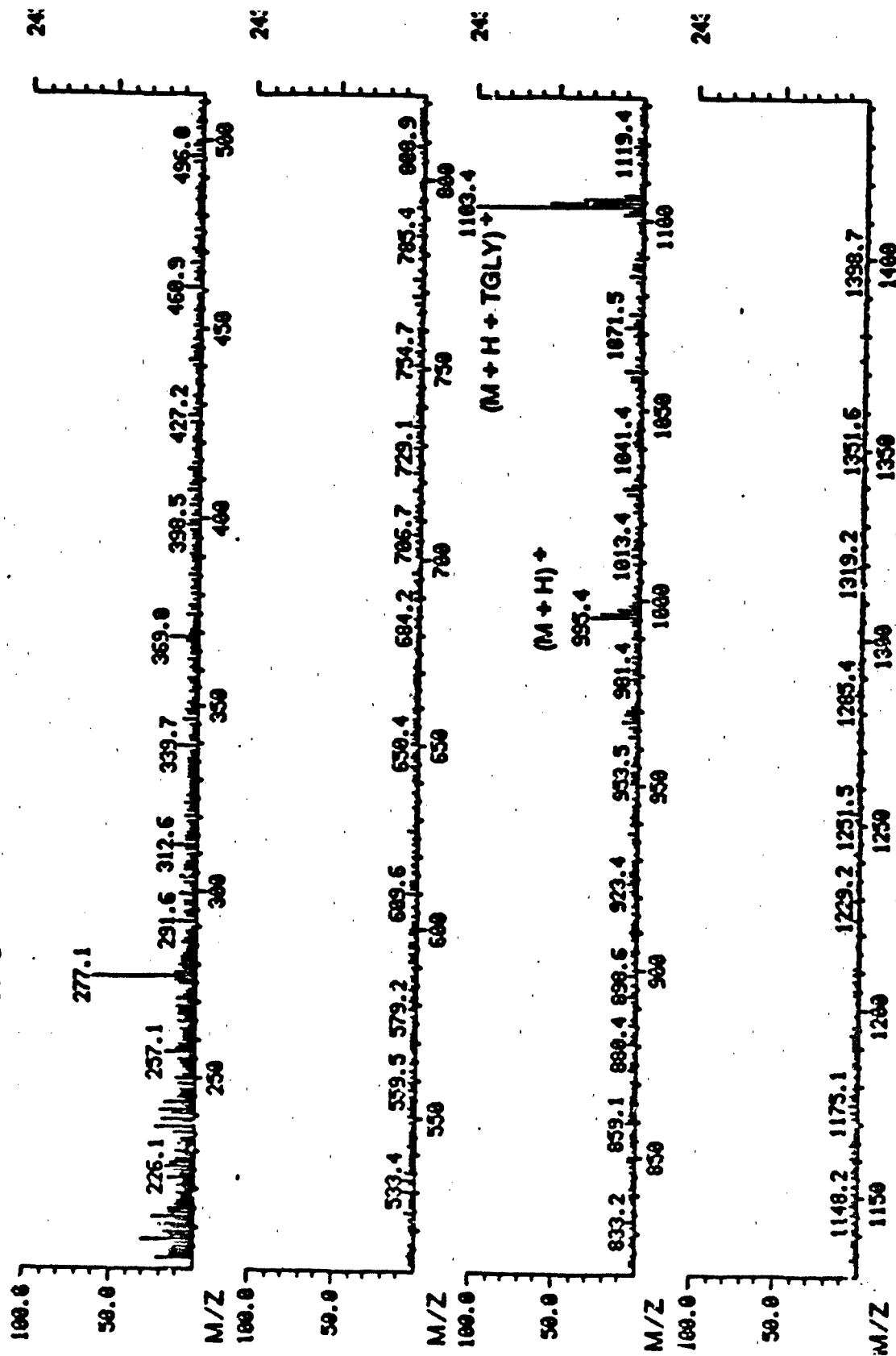
SAMPLE: 7620

CONDS: +/CI - KR/Q1 MASS SPECTRUM/7KV 12UA

GC TEMP: 149 DEG. C

DATA: HTK3 #31  
CALL: HCAL1127 #1

BASE M/Z: 1103  
RIC: 88192.



APPENDIX B

PAPER PRESENTED BY W. CARMICHAEL AT 6TH INTERNATIONAL  
IUPAC SYMPOSIUM ON MYCOTOXINS AND PHYCOTOXINS  
PRETORIA, REPUBLIC OF SOUTH AFRICA  
JULY 22-25, 1985

\*"IN PRESS" - ELSEVIER, AMSTERDAM

\*In Mycotoxins and Phycotoxins. (P.S. Steyn and R. Vleggaar,  
eds.). (1986). Elsevier Science Publishers, Amsterdam. pp. 354-  
364.

PHYSIOLOGICAL EFFECT OF A PEPTIDE TOXIN PRODUCED BY THE FRESH-WATER CYANOBACTERIA (BLUE-GREEN ALGAE) MICROCYSTIS AERUGINOSA. STRAIN 7820

W.W. Theiss<sup>1,2</sup> and W.W. Carmichael<sup>1</sup>

<sup>1</sup>Department of Biological Sciences, Wright State University, Dayton, Ohio 45435 (U.S.A.)

<sup>2</sup>Current Address - Harbor Branch Laboratory; SeaPharm, R.R. 1, Box 496, Ft. Pierce, Florida 33450 (U.S.A.)

**ABSTRACT**

The hepatotoxin of Microcystis aeruginosa strain 7820 was found to be a cyclic peptide (M.W. 994) with an LD<sub>50</sub> i.p. mouse of 50-100 ug/kg. In time course pathology studies (2-3 h) using rats it was found that liver damage occurred prior to the appearance of pulmonary thrombi and in no animal was the thrombi extensive enough to be life-threatening. Anesthetized rats cannulated to measure pressure in the jugular vein, hepatic portal vein and the femoral artery showed no pressure patterns that would indicate venous congestion. Concurrent measurements of lactic acid and femoral arterial pressure showed a rise in lactic acid levels paralling the fall in systemic arterial pressure. Isolated rat liver perfusion experiments showed that the toxin has an effect which paralleled and mimicked that seen in whole animal studies. It was concluded that the toxin is a potent, rapid acting hepatotoxin, with the immediate cause of death being hemorrhagic shock secondary to hepatocellular necrosis and collapse of hepatic parenchyma.

**INTRODUCTION**

While hepatotoxic peptides are now known to be produced by strains of Anabaena flos-aquae, Microcystis aeruginosa, Oscillatoria agardhii and suspected in at least two other genera, the toxins of M. aeruginosa have been studied most (Carmichael, 1981; Carmichael, et al., 1985). Rats and mice injected with acutely toxic doses of M. aeruginosa cells or toxin extract die within 1 to 3 hours. Upon necropsy, the animals show grossly enlarged livers engorged with blood, with other organs being pale. Liver weight is increased, and at death composes about 8 to 10 percent of body weight of mice as compared to about 5 percent in controls (Falconer, et al., 1981; Slatkin et al., 1983). Histopathological examination of the liver reveals extensive centrilobular hemorrhagic necrosis with loss of characteristic architecture of the hepatic plates. The hepatocytes appear to have lost cell to cell adhesion. Gross histological examination of intestine, heart, spleen, kidneys and stomach show no consistent abnormalities; lungs are mildly congested with occasional intravascular



protein deposits, described as atypical granular thrombi, not attached to endothelia. Slatkin, et al., (1983) suggested that these thrombi may be a direct effect of the toxin, which leads to pulmonary congestion and right heart failure. This, in turn, could cause blood pooling and congestion in the liver. However, in time course studies, Falconer, et al., (1981) reported that the pulmonary occlusions only appeared in histological preparations taken after 30 minutes toxin injection, while liver damage was noted as early as 15 minutes. This observation and others have led investigators to suggest that the liver damage is a direct effect of the toxin on the hepatocyte membrane and that the immediate cause of death in acutely dosed animals is hemorrhagic shock (Runnegar and Falconer, 1982; Ostensvik, et al., 1981).

The consistent pathological findings in animals poisoned by Microcystis are the swollen, blood-engorged livers with extensive centrilobular to panlobular hemorrhagic necrosis and the mildly edematous lung containing multiple intravascular, protein deposits. This has led to two theories for the mode of action of Microcystis toxin, 1) the toxin directly affects the liver, and pulmonary intravascular deposits are protein emboli originating from massive hepatocellular necrosis, and 2) the lung deposits are actually thrombi which result from the activation of coagulation mechanisms. These pulmonary thrombi cause an increase in resistance in the pulmonary capillaries, which result in the right heart failure; venous congestion and centrilobular necrosis in the liver.

The objectives of our study were to:

- I. Study the mechanism of hepatic injury and the source of pulmonary emboli found in histopathological examination of animals poisoned with M. aeruginosa strain 7820. The possible mechanisms are:
  - A. Direct action on the liver with debris from the liver carried to the lungs through the pulmonary circulation, resulting in pulmonary emboli. (Emboli are not considered to be thrombi unless they are true blood clots, consisting of fibrin, platelets, and blood cells and are attached to blood vessel endothelia).
  - B. Direct action on the coagulation process, causing either:
    1. Pulmonary thrombi and congestion, with subsequent right heart failure resulting in congestion and centrilobular necrosis of the liver, or
    2. Simultaneous blockage of outflow of hepatic vein by thrombi (Budd-Chiari Syndrome) and pulmonary

occlusion by thrombi.

II. Determine the cause of death in the acute toxic syndrome. Possibilities to be tested were as follows:

- A. Right heart failure
- B. Shock secondary to massive hemorrhage into the liver

#### METHODS

The cyanobacteria used for all testing was the single cell clonal isolate of *M. aeruginosa*, strain 7820. The strain was obtained in 1976, from Loch Balgavies, near Forfar by Dundee, Scotland, and is maintained in the Pasteur Institute collection (Codd and Carmichael, 1982). Freeze-dried cells have an IP LD<sub>50</sub> of approximately 50 mg/kg in mice with survival times of 30 to 120 minutes. Toxin was extracted by a modified method of Siegelman, et al., (1984) (Carmichael, unpublished) data. This involves aqueous methanol/butanol extraction of the lyophilized cells, centrifugation and separation of plant pigments with C-18 silica gel cartridges (Analytichem Bond Elute®). This is followed by Sephadex G-25 gel filtration with a final purification of the toxin using high performance liquid chromatography on a semi-preparative (9.4 mm x 25 cm) C-18 column. The purified toxin has an intraperitoneal mouse LD<sub>50</sub> of 50 ug/kg. Each gram of lyophilized cells yields about 1-2 mg of toxin which is a recovery of between 70-75%.

Renal studies were conducted using Sprague-Dawley rats injected with sublethal doses of a saline cell suspension and kept in Hoeltge metabolism cages. Urine was collected and tested every twelve hours for three days to obtain baseline values before administration of the toxin. Chemical tests were done for glucose and hemoglobin, utilizing the Ames N-Multistix (Miles Laboratories). Urine sediment was examined for casts, white blood cells, red blood cells, crystals, and epithelial cells.

In time-course pathology studies rats were injected intraperitoneally with 1200 ug extract/kg which gave a survival time of 90 to 150 minutes (Fig. 1). Rats were sacrificed at 20, 40, and 60 minutes after injection. One rat was allowed to die from the effects of the toxin, which occurred at 90 minutes; a control was injected with saline and sacrificed at 90 minutes. Trachea, esophagus, lungs, heart, spleen, thymus, kidneys, adrenals, pancreas, jejunum, colon, stomach, testes, seminal vesicles, and prostate were taken from the 90-minute rat and the control rat; only lungs and liver were taken from the others. The organs were fixed sectioned, and stained with hemotoxylin and eosin (H&E) or phosphotungstic acid and hematoxylin (PTAH). Male ICR/Swiss mice were injected intraperitoneally with 200 ug extract/kg body weight and sacrificed by halothane inhalation at 15, 30, 45, and 60 minutes. One mouse was allowed to die from the effects of the

toxin after one hour and a saline injected control was sacrificed at one hour. Organs removed for microscopic examination were the same as those for the rat studies.

Rat arterial and venous blood pressures were measured by cannulation of blood vessels with Intramedic polyethylene tubing (Clay Adams). Non-fasted animals were anaesthetized for the surgery by intraperitoneal injections of sodium pentobarbital. 50 mg/kg, and maintained under anaesthesia by injection directly into the arterial catheter. The animal was monitored for at least 30 minutes to obtain baseline values before injection of the toxin via the arterial catheter.

For lactic acid studies, blood (0.2 mL) was drawn from a femoral arterial catheter and a perchloric acid protein-free filtrate was prepared. Lactic acid levels of the protein-free filtrates were determined using a Sigma Kit No. 826-UV (Sigma Chemical Company).

For isolated liver perfusion studies rat livers were perfused with a hemoglobin-free medium consisting of a perfluoro-tributylamine emulsion as oxygen-carrier (Fluosol-43; Alpha Therapeutic Corporation) in a Krebs-Ringer bicarbonate solution adjusted to pH 7.4 with 0.1 M  $\text{NaHCO}_3$ . The perfusion apparatus used for these studies was maintained at the Naval Medical Research Institute-Toxicology Detachment, Wright Patterson AFB, Ohio. The perfusate was oxygenated initially with a mixture of 95% oxygen - 5% carbon dioxide until just before the isolated liver was placed in the circuit, at which time 100% oxygen was introduced and continued throughout the remainder of the experiment. The flow of the oxygen was adjusted to maintain the pH of the media between 7.35 and 7.45. Livers were removed and prepared for perfusion by the surgical technique first described by Hems, et al., (1966). The liver was transferred on nylon mesh to the perfusion chamber and connected to the circulating medium. A constant perfusion rate of 25 mL/min was maintained throughout the experiment. Sodium taurocholate (.56  $\mu\text{mole/min}$ ) was infused into the circulating media to ensure adequate salts for bile production. After completion of the perfusion, the liver was removed, flushed with ice-cold 0.15 M KCL and then 10% formalin solution.

## RESULTS

Intraperitoneal  $\text{LD}_{50}$  values for mice were determined on each batch of lyophilized algae and each toxin extraction.  $\text{LD}_{50}$  values for both rats and mice were determined on one large batch of purified toxin. Mouse  $\text{LD}_{50}$  for the lyophilized material was approximately 50 mg/kg. The  $\text{LD}_{50}$  for both rats and mice on purified toxin was approximately 50  $\mu\text{g/kg}$  (1000X purification). There were differences in survival times for rats and mice. Mice given lethal doses almost invariably died within one-half to two

hours, and on rare occasions at low doses, survived for up to four hours (data not shown). If the mice survived this period, they recovered completely. Rats showed a definite dose response regarding survival time, with animals surviving up to forty hours (Fig. 1) Rats surviving past forty hours completely recovered. While rats given the same dose of toxin in the high ranges ( $> 50$  ug/kg) showed little variation in survival time, rats given doses of 50 ug/kg and below showed considerable variation in survival times. Survival time was two hours at a dose of 400 ug/kg and further increases in dosage, up to 3200 ug/kg, did not significantly shorten rat survival time.

In renal studies, no glucose, casts, leukocytes, erythrocytes, epithelial cells, or any other abnormal constituents were found in the urine with the exception of hemoglobin, resulting from hemorrhage in the liver which released large amounts of hemoglobin into the circulation. Histopathological examination of the kidneys of rats dosed with 7820 toxin showed no differences when compared to kidneys of control rats.

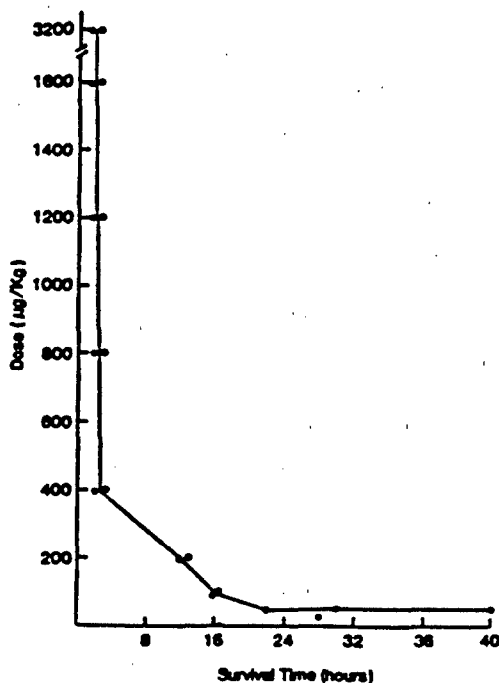


Fig. 1. Survival time of male rats (250-300g) dosed with *M. aeruginosa* 7820 toxin. Doses  $> 400$  ug/kg did not further shorten survival time.  $LD_{50}$  was approximately 50 ug/kg. Mice did not show a similar survival time/dose response, but  $LD_{50}$  was the same as rats. Each data point represents one animal.

Complete necropsy performed on rats and mice dying from the effects of the toxin revealed liver and lung effects previously reported by other researchers. No abnormalities were noted in any other organs. Grossly the liver appeared swollen and blood engorged, with liver weights comprising approximately 8%-10% of the animal's total body weight rather than the 5% observed in controls. Lungs exhibited mild edema.

Microscopic examination of H & E stained liver sections of rats and mice revealed a centrilobular to panlobular hemorrhagic necrosis. There was acute, diffuse, moderate to severe congestion and complete disassociation of the hepatocellular cords. Many hepatocytes exhibited acute cellular swelling and cytoplasm had been converted into a homogeneous, eosinophilic mass. Hepatocyte nuclei were frequently swollen, but contained evenly dispersed chromatin. Many central veins and disrupted sinusoids contained red blood cells and mixed with amorphous, globular, eosinophilic debris thought to be of hepatocellular origin. The morphologic appearance of this debris appeared very similar to that of the degenerating hepatocytes. In some cases, this amorphous debris appears to be associated with deposits of pale-staining fibrillar material that could be fibrin. Occasional central veins contain dislodged degenerating hepatocytes.

The pulmonary vessels of rats and mice contained multiple deposits of amorphous, globular eosinophilic deposits which were not distinctly attached to endothelial cells. This intravascular debris was morphological compatible with proteinaceous debris noted in liver sinusoids and hepatic central veins. PTAH staining of sections indicated the absence of fibrin in the deposits. True thrombi, or blood clots, consist of aggregated platelets with fibrin and trapped cells and they are usually attached to the endothelia. Because of the small size and infrequent numbers of the pulmonary emboli, they were not thought to be life-threatening in either the rat or the mouse, and probably consisted of degenerating hepatocytes and other debris transported from the liver to the pulmonary capillary beds. In time course studies of the mouse, liver lesions were apparent within 15 minutes, but no pulmonary emboli were noted until one hour after administration of the toxin. In rats, both liver lesions and emboli were noted at 20 minutes. Liver lesions were moderately severe and numerous, but emboli were mild and minimal.

Systemic arterial pressure and blood lactic acid concentrations were monitored in anaesthetized control rats and during the course of acute toxicity (Fig. 2). The blood pressure slowly decreased to values indicative of hypovolemic shock (40-50 mm Hg) and continued decreasing until the animal died (20 mm Hg). Lactic acid levels, an indicator of anaerobic metabolism, showed a constant increase that paralleled the decrease in arterial blood pressure, thus verifying that circulation to the organs and tissues was not adequate to maintain normal aerobic metabolism.

This condition is defined as shock.

Measurements of jugular venous and hepatic portal pressures, along with systemic arterial pressures (Figs. 3-4), revealed the typical declining arterial pressure, but no significant changes were noted in either jugular pressure or hepatic portal pressure during the course of toxicity. Pulmonary emboli of sufficient severity to cause right heart failure and centrilobular liver necrosis should cause venous hypertension. Jugular and hepatic portal venous pressure would rise to levels above 10 mm Hg.

Isolated, perfused rat livers, after a 30 minute period to obtain baseline bile flow values, were treated with purified toxin. Upon introduction of the toxin into the perfusate media, bile flow immediately slowed and completely ceased within 10 minutes. After approximately 30 to 40 minutes, the liver was visibly swollen with accumulation of perfusate media. Livers were tested at equal doses with and without the Fluosol in the Krebs-Ringer solution, and no differences were noted in bile flow and the toxic sequelae.

Histopathologic examination of toxin-treated perfused liver revealed complete disassociation of hepatocytes with obliteration of normal lobular architecture and effacement of sinusoidal spaces (Figs. 5-6). Hepatocytes were round and usually exhibited intensely eosinophilic, homogeneous cytoplasm. In some liver cells, however, the cytoplasmic contents displayed an eosinophilic, finely granular texture. Most hepatocellular changes were consistent with hepatocellular albuminous degeneration.

#### DISCUSSION

The cause of death due to the toxin of *M. aeruginosa*, strain 7820, in rats treated with purified toxin, appeared to be hypovolemic shock, as indicated by the steady fall in systemic arterial blood pressure, coupled with the parallel rise in blood lactic acid concentration. Hypovolemic shock was caused by interstitial hemorrhage into the liver since complete gross and histopathological examination revealed no other sites of hemorrhage in the body. The increase in liver weight of the treated rats accounted for about 50% of the original blood volume of the animals. Loss of 30% to 40% of the blood volume within a two hour period is adequate hemorrhage to produce irreversible hypovolemic shock (Guyton, 1976). Blood pressures have not been measured in mice, but mice showed an increase in liver weight similar to that in rats.

Disseminated protein deposits in pulmonary vessels noted microscopically were not regarded as thrombi (blood clots) consisting of fibrin and cells attached to the endothelia. Instead, they appeared to be emboli consisting of protein debris liberated from the necrotic hepatocytes. Emboli were not

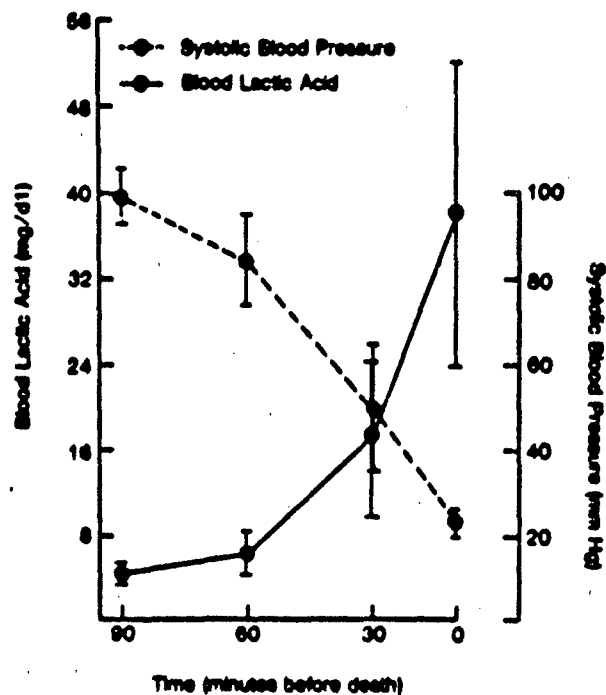


Fig. 2. Systolic femoral arterial blood pressures and blood lactic acid concentrations of anaesthetized rats dosed with Microcystis (strain 7820) toxin, 1200 ug/kg IV, (N=4). Blood pressure gradually decreased, causing first shock, and then death. Lactic acid rose as the blood pressure fell. Animals died when blood pressure fell to levels below 20 mm HG. Blood lactic acid concentrations at death showed a high variation among the animals tested. Lactic acid and arterial pressure were plotted with respect to time before death rather than time after toxin administration due to variation in the time of onset of fall in arterial pressure and the rise in lactic acid. Control rats (N=3) showed no significant change in blood pressure.

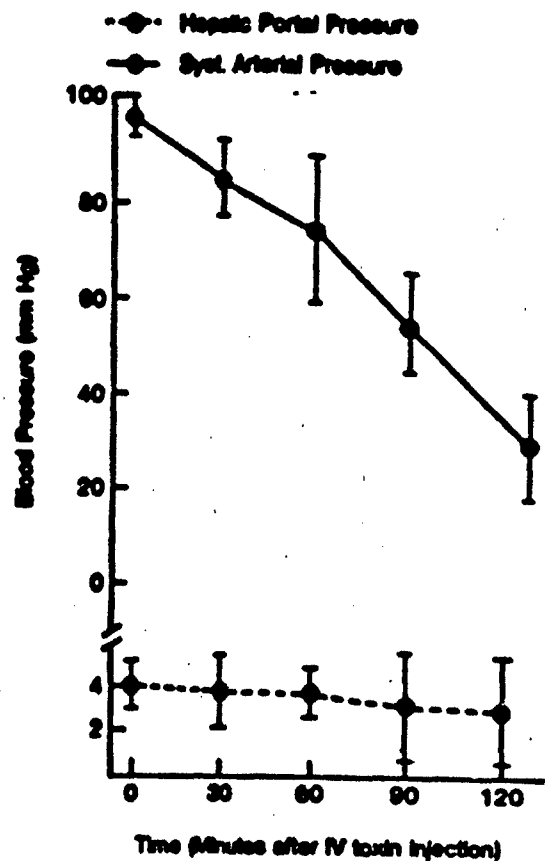
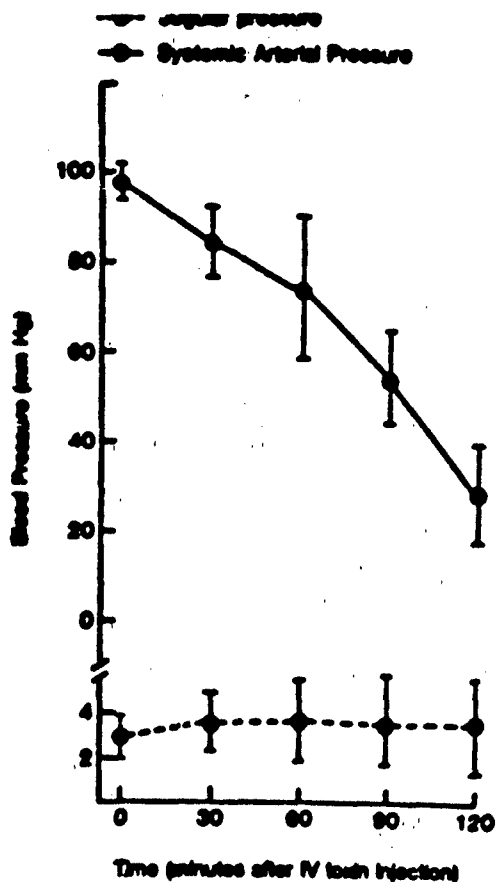


Fig. 3. Femoral arterial and jugular venous pressures of rats dosed IV with Microcystis (strain 7820) toxin (N=8). Arterial pressure steadily fell to shock levels, but jugular venous pressure was stable and normal, an indication of the absence of venous congestion and right heart failure. Animals died when arterial pressure fell to 20 mm HG. This normally occurred between 90 and 120 minutes after toxin administration. Control rats (N=3) showed no significant change in blood pressure.

Fig. 4. Femoral arterial pressures and hepatic portal venous pressures of rats dosed IV with Microcystis (strain 7820) toxin, 1200 ug/kg (n=8). Arterial pressure steadily fell to shock levels, but hepatic portal pressure was stable, with no evidence of venous congestion and right heart failure. Animals died when arterial pressures fell to 20 mm HG. This normally occurred between 90 and 120 minutes after toxin administration. Control rats (N=3) showed no significant change in blood pressure.



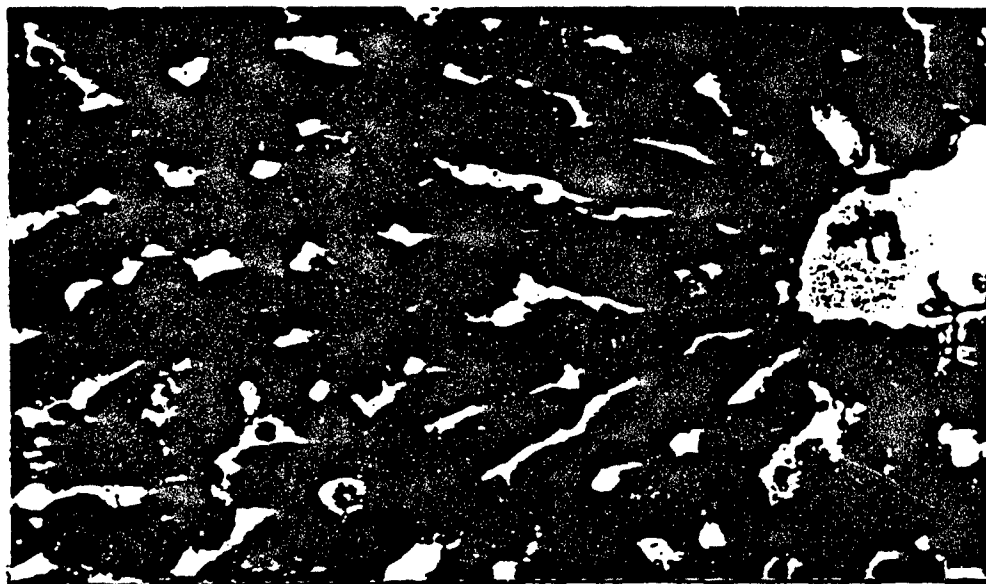


Fig. 5. Control isolated, perfused rat liver after 90 min. perfusion with fluosol. Note normal architecture of hepatocyte plates, sinusoids and central vein (right center). [100X]

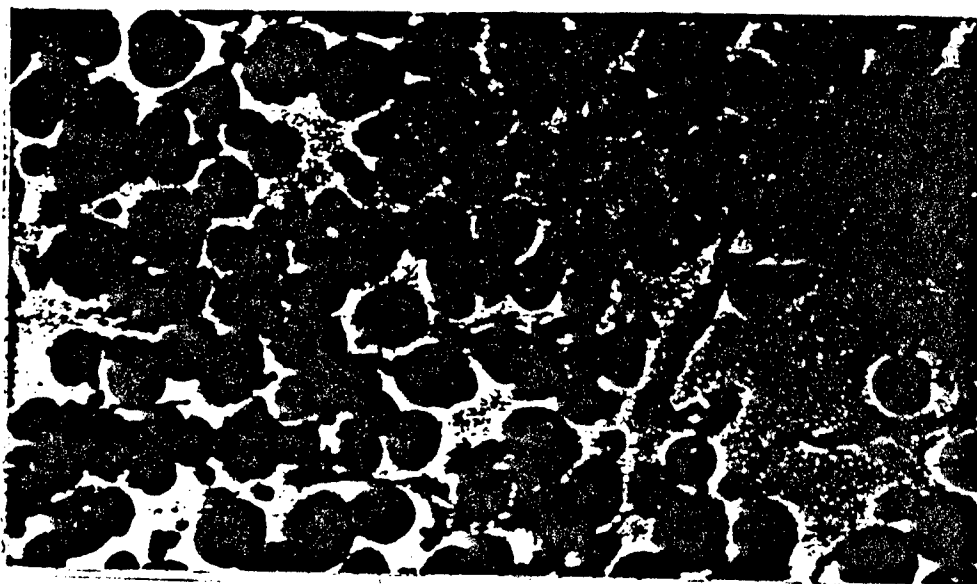


Fig. 6. Typical section of isolated, perfused rat liver after 30 min. perfusion with fluosol followed by a 60 min. perfusion with fluosol plus 200 ug of toxin (2 ug/ml of perfusate). Note complete panlobular disruption of normal lobular architecture. Hepatocytes are distorted and swollen. [100X]

attached to endothelia. The pulmonary capillary beds serve as "traps" for debris in the veins since they are the first small vessels encountered in the circulation after liberation of debris into the venous system of the liver (Robbins and Cotran, 1979).

The emboli did not cause death, right heart failure, or liver necrosis. The lesions did not appear to be of adequate severity to kill either the rats or mice, and in the mouse did not appear until after liver lesions were noted. Emboli appeared earlier in rats than in mice, but were minimal. Measurements of jugular and hepatic portal venous pressures indicated no venous congestion. If pulmonary congestion had caused right heart failure and right heart failure had subsequently been the cause of the centrilobular to panlobular liver necrosis, venous pressures would have increased to levels of at least 10 mm HG or more, probably as high as 20 to 30 mm HG. Liver necrosis due to right heart failure, as observed in humans, usually occurs over a much longer time course, days and weeks rather than one to two hours (Robbins and Cotran, 1979).

The suggestion by Slatkin, et al., (1983) that death from Microcystis toxin could be due to right heart failure was based on presence of atypical thrombi in the pulmonary capillaries of mice sacrificed, when moribund, during a period of 60 to 117 minutes after dosing, as compared to the absence of these atypical thrombi in the pulmonary capillaries of two surviving mice given sublethal doses of toxin (lower than any of the mice sacrificed when moribund) and sacrificed 22 and 72 hours after dosing. However, the survival of these animals and the absence of emboli in their lungs could have been due to a common cause: inadequate liver damage to produce lethal hemorrhage or the deposition of debris into the venous system, rather than the presence of atypical thrombi causing death. Had emboli been present during the first one to two hours after injection of toxin in these low-dose, surviving animals, as were noted in the moribund animals, they probably would have been removed by lysis or leukocyte phagocytosis before the animals were sacrificed.

Falconer, et al., (1981) noted the presence of thrombi in histological sections of mice 60 minutes after IP toxin injection, well after liver lesions were noted. It was suggested that the death in these acutely poisoned mice might be related to pulmonary circulatory failure due to shock and the development of disseminated intravascular coagulation within the lungs. However, criteria for classifying the lung lesions as thrombi were not stated and no lesions described as thrombi could be found in other organs. Disseminated intravascular coagulation would have produced thrombi elsewhere, especially in the kidneys.

There is no evidence that the toxin has any direct effect on the coagulation processes of the body, even though that possibility cannot be ruled out. No substantial number of lesions that

could be definitely described as thrombi were found anywhere in the body in our study or previous studies (Falconer, et al., 1981; Slatkin, et al., 1983) of Microcystis toxicity. Administration of the anticoagulants heparin, acetylsalicylic acid, Malayan pit viper venom, streptokinase, and warfarin in a previous study by Slatkin, et al., (1983) did not affect the peptide toxicity. In this same study, no differences were noted in whole blood clotting time, euglobulin lysis time, prothrombin time, activated partial thromboplastin, and fibrinogen in control and toxic animals. Suspicion of an effect on the coagulation system was based on the disappearance of 80% of the circulating platelets during the course of toxicity and the assumption that pulmonary emboli were atypical thrombi resulting from some coagulation process. As explained above, the majority of the pulmonary emboli were not true thrombi. There is no evidence of the fate of these platelets. They could have been consumed at the site of injury and hemorrhage in the liver. Severe trauma, hemorrhage, endothelial injury and/or exposure of platelets to collagen will cause platelet aggregation (Guyton, 1976; Robbins and Cotran, 1979). Therefore, the disappearance of platelets from the circulation may have been a secondary effect of the liver injury. Future studies should more thoroughly examine the effects of the toxin on platelets both in vivo and in vitro.

It was further concluded that the toxin had a direct effect on the liver, not mediated by any other system in the body, as indicated in the isolated, perfused liver studies. The effect was rapid, as noted by the prompt cessation of bile flow when the toxin was added to the perfusate media. Visible swelling of the isolated liver was noted 30 to 40 minutes after addition of the toxin, corresponding to the swelling noted in vivo. Histopathological examination of the liver after 60 minutes of exposure to the toxin revealed disruption of normal liver architecture similar to that noted microscopically in livers of animals exposed by IP injection. The absence of blood and any clotting factors in the perfusate media ruled out the necessity of any mediation of liver injury by the coagulation system.

Blockage of venous circulation in the liver, called Budd Chiari syndrome, may also cause centrilobular liver necrosis. This mechanism has been proposed for the toxic action of pyrrolizidine alkaloids found in tansy ragwort (Senecio) (McLean, 1970). These toxins produce portal hypertension accompanied by centrilobular, hemorrhagic necrosis that is usually fatal about three days after injection of a lethal dose. The rapid action and lack of hepatic portal hypertension ruled out this mechanism of hepatotoxicity for Microcystis.

The hepatocyte appeared to be the most likely cell candidate for toxic action, even though connective tissue and sinusoidal endothelia cannot be ruled out as targets. Acute panlobular hepatocellular swelling, an indicator of cell membrane damage, is

noted. Bile synthesis is primarily a function of the hepatocyte and even though damage to connective tissue or endothelial could have interrupted it eventually, it is doubtful that injury to any cell other than the hepatocyte, could cause complete cessation in such a short time. Interruption of bile flow is a somewhat non-specific indicator of hepatocyte damage, and little can be learned as to the specific mechanism of action on the cell. Changes in membrane permeability, changes in enzyme activity, interruption of protein synthesis, blockage of transport of bile salts or blockage of outflow of bile could all cause cessation of bile flow. Cellular swelling and disassociation, as noted in Microcystis, will disrupt bile canaliculi immediately. Tests of viability of exposed hepatocytes, such as growth in culture, could provide a clue to the actual damage rendered to the hepatocyte.

Numerous possibilities exist as to the cellular mechanism of action of the toxin. Runnegar, et al., (1981) suggests that the specificity of the toxin for the liver may be due to transport of the toxin into the hepatocyte by the bile acid transport mechanism. This assertion was based on prevention of toxin deformation of isolated hepatocytes by the addition of sodium deoxycholate, a bile salt, to the incubation media. Further studies are needed, however, to determine what relationship exists between Microcystis hepatotoxicity and the bile transport mechanism.

#### ACKNOWLEDGEMENTS

The technical assistance of the following people is greatly appreciated: H. Bruner (Pathology); M. Mullins (blood pressure); J. Wyman (liver perfusions).

## REFERENCES

- Carmichael, W.W., (Editor), 1981. The Water Environment: Algal Toxins and Health. Plenum Press, New York, 491 pp.
- Carmichael, W.W., Jones, C.L.A., Mahmood, N.A. and Theiss, W.C., 1985. Algal Toxins and Water-based Diseases. CRC Reviews in Environmental Control, 15: 275-313.
- Codd, G.A., and Carmichael, W.W., 1982. Toxicity of a clonal isolate of the cyanobacterium Microcystis aeruginosa from Great Britain. FEMS Microbiol. Letters, 13: 409-411.
- Falconer, I.R., Jackson, A.R.B., Langley, J. and Runnegar, M.T., 1981. Liver pathology in mice in poisoning by the blue-green alga Microcystis aeruginosa. Aust. J. Biol. Sci., 34: 179-187.
- Guyton, A.C., 1976. Textbook of Medical Physiology. W.B. Saunders Co., Phil., pp. 357-364.
- Hems, R., Ross, B.D., Berry, M.N. and Krebs, H.A., 1966. Gluconeogenesis in the perfused rat liver. Biochem. J., 101: 284-292.
- McLean, E.K., 1970. The toxic actions of pyrrolizidine (Senecio) alkaloids. Pharmacol. Rev., 22: 429-448.
- Ostensvik, O., Skulberg, O.M. and Soli, N.E., 1981. Toxicity studies with blue-green algae from Norwegian inland waters. In: W.W. Carmichael (Ed.), The Water Environment: Algal Toxins and Health. Plenum Press, New York, pp. 315-324.
- Robbins, S.L. and Cotran, R.S., 1979. Pathologic Basis of Disease. W.B.S. Saunders Co., Philadelphia, pp. 814-875; 1000-1071.
- Runnegar, M.T.C. and Falconer, I.R., 1981. Isolation, characterization, and pathology of the toxin from the blue-green algae Microcystis aeruginosa. In: W.W. Carmichael (Ed.), The Water Environment: Algal Toxins and Health. Plenum Press, New York, pp. 325-342.
- Runnegar, M.T.C. and Falconer, I.R., 1982. the in vivo and in vitro biological effects of the peptide hepatotoxin from the blue-green alga Microcystis aeruginosa. S. Afr. Sci. 75: 363-366.
- Runnegar, M.T.C., Falconer, I.R. and Silver, J., 1981. Deformation of isolated rat hepatocytes by a peptide hepatotoxin from the blue-green alga Microcystis aeruginosa. Naunyn-Schieberg's Arch. Pharmacol., 317: 268-272.

Siegelman, H.W., Adams, W.H., Stoner, R.D. and Slatkin, D.N., 1984. Toxins of Microcystis aeruginosa and their hematological and histopathological effects. In: E.P. Ragelis (Ed.), Seafood Toxins. American Chem. Soc., Washington, D.C., pp. 407-413.

Slatkin, D.N., Stoner, R.D., Adams, W.H., Kycia, J.H. and Siegelman, H.W., 1983. Atypical pulmonary thrombosis caused by a toxic cyanobacterial peptide. *Science*, 220: 1382-1385.

**APPENDIX C**  
**CURRICULUM VITAE**  
**DR. ARUN S. DABHOLKAR**

**NAME:** ARUN S. DABHOLKAR

**QUALIFICATIONS:**

Ph.D. 1977, University of Udaipur, India, Zoology (Cytochemistry)  
M.S. 1964, University of Bombay, India, Zoology (Cytology)  
B.S. 1962, University of Bombay, India, Biology

Ph.D. Dissertation entitled 'Histochemical studies on the distribution of enzymes in the nervous system of invertebrates, vertebrates, and leprosy patients'.

**EXPERIENCES:**

1985-Present	Postdoctoral Research Associate	1. Electron microscopic studies of effects of hepatotoxin from blue-green algae.
1982-1984	Electron Microscopist/ Research Associate, Dept. of Oral Anatomy, Dental College, Univ. of Ill. Chicago, Ill. 60612	1. Electron microscopic studies of myotendon junctions in muscular dysgenic mice.
1979-1981	Research Associate, Dept. of Anatomy Northwestern Univ. Medical School, Chicago, Ill. 60611	1. Morphometry of synapses.
1977-1979	Research Associate, Neurology Service, V.A. Lakeside Medical Center & Dept. of Neurology, Northwestern Univ. Medical School, School, Chicago, Ill.	1. Electron microscopic and cytochemical study of the rat ventral prostate.
1974-1976	Japan Society for the Promotion of Science Fellow Dept. of Anatomy, Kansai Med. School, Osaka and Kyoto Univ. Med. School Kyoto, Japan	1. Ultrastructural morphology and electron microscopic cytochemistry of neurons from thiamine deficient rats.
1973-1974	Asst. Research Officer, Neuropathology Unit, J. J. Group of Hospitals and Grant Med. College,	1. Histochemistry of nerves and skin lesions from leprosy patients.



**Bombay, India**

- |           |   |  |
|-----------|---|--|
| 1970-1973 | Research Asst., Neuro-pathology Unit, J.J. Group of Hospitals and Grant Med. College, Bombay, India | <ol style="list-style-type: none"><li>2. Histology of nerves and skin lesions from leprosy patients.</li><li>3. Electron microscopy of lesions from leprosy patients.</li><li>4. Histochemistry of cells from tissue cultures of tumors.</li></ol> |
| 1968-1970 | Lecturer, Dept. of Zoology, Univ. of Udaipur, Udaipur, India  | <ol style="list-style-type: none"><li>1. Teaching Zoology for B.S. and M.S. degree students.</li></ol>   |
| 1966-1968 | Council of Scientific & Industrial (CSIR) Fellow, Dept. of Zoology Univ. of Udaipur, Udaipur, India | <ol style="list-style-type: none"><li>1. Histochemical studies on the distribution of enzymes in the nervous system of invertebrates &amp; vertebrates.</li><li>2. Teaching Zoology for B.S. students.</li></ol>                                   |
| 1964-1965 | Research Fellow, Biophysics Dept., Cancer Research Inst., Bombay, India                             | <ol style="list-style-type: none"><li>1. Irradiation of animals for induction of leukemia.</li><li>2. Routine histological and haematological techniques.</li><li>3. Cell fractionation by differential centrifugation.</li></ol>                  |
| 1962-1964 | Laboratory Instructor, Dept. of Biology Univ. of Bombay, Bombay, India                              | <ol style="list-style-type: none"><li>1. Taking laboratory courses for undergraduate students.</li></ol>   |

**MEMBERSHIPS:**

American Association of Anatomists  
Electron Microscopy Society of America  
New York Academy of Sciences

#### **PUBLICATIONS: ABSTRACTS**

Lakars, T.C., Herring, S.W. and Dabholkar, A.S.: Myotube structure during myogenesis in muscular dysgenic mice. Midwest Regional Developmental Biology Conference, W. Lafayette, 1983.

Dabholkar, Arun S.: Hepatic peroxisome membrane: freeze-fracture study. The Anatomical Record 205: 1983, 96th annual session of American Association of Anatomists, Atlanta, 1983.

Lakars, T.C., Herring, S.W. and Dabholkar, A.S.: Development of the muscle-tendon junction in normal and muscular dysgenic mice. The Anatomical Record 205: 1983, 96th annual session of American Association of Anatomists, Atlanta, 1983.

Dabholkar, Arun S.: Vitamin E deficiency and hepatic peroxisomal biology. The Anatomical Record 202: 1982, 95th annual session of American Association of Anatomists, Indianapolis, 1982.

Dabholkar, Arun S.: Peroxisomal proliferation in the hepatocytes of vitamin E deficient rats; electron microscopic study. The Anatomical Record 199: 1981, 94th annual session of American Association of Anatomists, New Orleans, 1981.

Dabholkar, Arun S.: Vitamin E deficiency as hepatic peroxisomal proliferator: electron microscopic evidence. International Conference on Peroxisomes and Glyoxysomes, New York, 1981.

Pysh, J.J. and Dabholkar, A.S.: Ultrastructure of B and C synaptic terminals in sympathetic ganglion. The Anatomical Record 196: 1980, 93rd annual session of American Association of Anatomists, Omaha, 1980.

Dabholkar, Arun S.: Lysosomal distribution in neurons from the cerebrum and spinal cord during early vitamin E deficiency: electron microscopic study. 10th annual meeting of Society for Neuroscience, Cincinnati, 1980.

Koenig, H., Chung, Y.L. and Dabholkar, A.S.: Rapid effects of androgens on structure and secretion in rat ventral prostate: Ultrastructural and biochemical observations. The Anatomical Record 193: 1979, 92nd annual session of American Association of Anatomists, Miami, 1979.

Koenig, H. and Dabholkar, A.S.: Rapid effects of testosterone, pilocarpine and castration in rat ventral prostate: an electron microscopic and cytochemical study. Proc. of the 9th International Congress on Electron Microscopy, Toronto, Vol. II, p. 692, 1978.

Dastur, D.K., Porwal, G.L., Dabholkar, A.S., Lalitha, V.S., Ramamohan, Y., Shah, J.S. and Revenkar, C.R.: Lysosomal activity of Schwann cells and macrophages in leprous neuropathy: Histochemistry and electron microscopy; VIIIth International Conference on Neuropathology, Washington, D.C., 1978.

Dabholkar, Arun S. and Ogawa, K.: Ultracytochemical changes in the distribution of acid phosphatase and thiamine pyrophosphatase activity in the nerve cells of vitamin E deficient rats. *Acta Histochem. Cytochem.* 10: 148, 1977 and Proc. of the 17th annual meeting of the Japan Society of Histochem. & Cytochem., Tokyo, 1974.

Dabholkar, A.S. and Ogawa, K.: Ultrastructural changes in the distribution of acid phosphatase and thiamine pyrophosphatase activity in the nerve cells of the cerebrum of thiamine deficient rats. *Acta Histochem. Cytochem.* 9: 89, 1979 and Proc. of the 16th annual meeting of the Japan Society of Histochem. & Cytochem., Okayama, 1975.

#### PUBLICATIONS: PAPERS

Pysh, J. and Dabholkar, A.S.: Ultrastructure of B and C synaptic terminals in sympathetic ganglion of bull frog. (in preparation)

Dabholkar, Arun S.: Vitamin E deficiency as hepatic peroxisomal proliferator: electron microscopic evidence. *Cell Biol. Int. Reps.* 5: 851-855, 1981.

Reddy, J.K., Lalwani, M.D., Dabholkar, A.S., Reddy, M.K. and Qureshi, S.A.: Increased peroxisomal activity in the liver of vitamin E deficient rats. *Biochem. Int.* 3: 41-49, 1981.

Dabholkar, Arun S.: Vitamin E deficiency as hepatic peroxisomal proliferator; Qualitative and quantitative electron microscopic study. In: *Peroxisomes and Glyoxysomes*, H. Kindle and P.B. Lazarow, Eds., Annals New York Acad. Sciences: 386: 475-477, 1982.

Dabholkar, A.S. and Ogawa, K.: Ultracytochemical changes in the distribution of acid phosphatase in the liver cells of vitamin E deficient and vitamin E supplemented rats. *Acta Histochem. Cytochem.* 11: 195-204, 1978.

Dabholkar, A.S. and Ogawa, K.: Ultracytochemical changes in the distribution of acid phosphatase and thiamine pyrophosphatase activity in the nerve cells of vitamin E deficient rats. *Acta Histochem. Cytochem.* 11: 52-63, 1978.

Dabholkar, A.S. and Tewari, H.B.: Histochemical studies on the distribution of adenosine triphosphatase and 5'-nucleotidase

among the constituents of the thoracic ganglia and the associated nerve of *Periplaneta americana*. *Acta Morphologica Neerlandoscand.* 16: 77-84, 1978.

Dabholkar, A.S. and Ogawa, K.: Ultracytochemical changes in the distribution of acid phosphatase and thiamine pyrophosphatase activity in the nerve cells of the cerebrum of thiamine deficient rats. *Acta Histochem. Cytochem.* 9: 214-226, 1976.

Dastur, D.K., Ramamohan, Y. and Dabholkar, A.S.: Some neuropathologic and cellular aspects of leprosy. *Progr. Res.* 18: 53-75, 1974.

Dastur, D.K. and Dabholkar, A.S.: Histochemistry of leprous nerves and skin lesions. Acid phosphatase. *J. Path.* 113: 69-77, 1974.

Dabholkar, A.S. and Tewari, H.B.: The functional significance of the presence of acid phosphatase at the nucleic-acid synthesizing sites in the nuclei of the neurons of the cephalothoracic ganglionic masses of *Palaemonetes*. *Acta Neurolog. Scand.* 44: 533-541, 1968.

Tewari, H.B. and Dabholkar, A.S.: Histochemical studies on the distribution of alkaline and acid phosphatase among the constituents of the abdominal and thoracic ganglia and the nerve cord of *Periplaneta americana*. *Acta Histochemica* 30: 350-363, 1968.

**APPENDIX D**

**TOXIN DATA BASE PROFILES:  
TOXINS OF BLUE-GREEN ALGAE (CYANOBACTERIA)**

**BY**

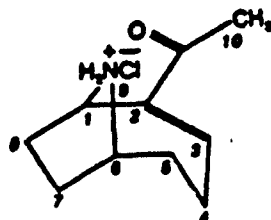
**WAYNE W. CARMICHAEL, PH.D.  
DEPT. OF BIOLOGICAL SCIENCES  
WRIGHT STATE UNIVERSITY**

**PREPARED FOR PUBLICATION IN  
Compendium of Natural Toxicants and Venoms-  
Marcel Dekkar (A. Ciegler, ed.)**

## ANATOXIN-A

1. Name: Anatoxin-a (2-acetyl-9-azabicyclo[4,2,1]non-2,3-ene)  
Ref: (Devlin et al., 1977; Huber, 1972)

2. Structure:



Ref: (Devlin et al., 1977; Huber, 1972)

3. Producing Biological Forms:

Anabaena flos-aquae (Lyngb.) de Breb. Strain NRC-44-1. A freshwater filamentous blue-green alga (Cyanobacteria) in the family Nostocaceae (Fig. 1). Ref: (Carmichael and Gorham, 1978; Gorham et al., 1964)

4. Physico-chemical Properties:

- a. Elemental - Anatoxin-a =  $C_{10}H_{15}NO$
- b. M.W. - Anatoxin-a = 165 (one HCl)
- c. M.P. - (N.A.)
- d. Optical Rotation - (N.A.)
- e. Spectroscopic - U.V. (hydrochloride) -  $\lambda$  max at 227 nm (ethanol)  $\epsilon = 10,000$ .  
Ref: (Devlin et al., 1977)
- f. Spectroscopic - I.R. (hydrochloride) -  $\lambda$  max ( $CHCl_3$ ) 2300-2800; ( $NH_2^+$ ) 1670 (str); 1645 (wk); 1588 (med)  $cm^{-1}$ .  
Ref: (Devlin et al., 1977)
- g. Spectroscopic - NMR (hydrochloride) - 60 MHz proton in deuterium oxide - one hydrogen signal at  $\delta$  7.1 (t,  $J=6$  Hz), 4.7 (d,  $J=6$  Hz) and 3.9 broad relative to a 3H singlet arbitrarily set at  $\delta$  2.0 ( $COCH_3$ ).  
Ref: (Devlin et al., 1977)
- h. Spectroscopic - Mass Spec. (hydrochloride) - (direct inlet  $98^\circ$ ) gives peaks at m/e (relative intensity):

166(22), 165(100), 164(9), 151(6), 150(28), 137(22),  
136(38), 123(10), 122(54), 108(12), 94(26), 94(26), 93(7),  
83(8), 82(31), 81(8), 69(31), 68(28), 67(10).  
Ref: (Devlin et al. 1977)

- i. Spectroscopic - Circular Dichroism - (N.A.)
- j. Solubility - Soluble in water, ethanol, methanol.
- k. X-ray Crystallography - (N-acetyl derivative) - Crystal data: space group  $P4_2,2,2$ ,  $D_m=1.19 \text{ gcm}^{-3}$ ,  $Z=8$ ,  $D_c=1.21 \text{ gcm}^{-3}$ ,  $a=7.335(2)$ ,  $c=42.294(10) \text{ \AA}$   
Ref: (Huber, 1972)
- l. Crystal Description - Tetragonal uniaxial colorless prisms of square cross-section - approximate dimensions  $0.25 \times 0.25 \times 0.22 \text{ mm}$ .  
Ref: (Huber, 1972)
- m. Specific Gravity - (N.A.)
- n. Misc. Properties - Acid, light and heat stable; alkaline labile; readily dialyzable.

5. Chemistry:

- a. Derivatives - n-acetyl; iso; dihydro derivatives have been formed  
Ref: (Devlin et al., 1977)
- b. Chemical Synthesis - accomplished by:
  1. ring expansion of cocaine (Campbell et al., 1977);
  2. synthesis from 1,5-cyclooctadiene (Campbell et al., 1979);
  3. synthesis from 1-methylpyrrole (Bates and Rapoport, 1979).

6. Production:

A. flos-aquae NRC-44-1 is batch cultured ( $22-24^\circ\text{C}$ ) in ASM-1 medium (Carmichael, 1985) using continuous lighting from cool white fluorescent and Durotest<sup>®</sup> Vitalight bulbs. Light intensity is  $70-90 \text{ uEin/m}^2/\text{sec}$  of photosynthetically active radiation as measured at the culture surface by a Lambda LI-80 meter and quantum sensor. Cultures are harvested at the stationary phase of growth (12-15 days) when the cell counts are  $4-6 \times 10^6 \text{ cells ml}^{-1}$ . Harvesting is done using a Millipore Pellicon cell concentrator. Cells are lyophilized and the dried cells stored at  $-80^\circ\text{C}$  until used for toxin extraction. An average of 100 mg/liter dried cells are produced. The  $\text{LD}_{50}$  intraperitoneal mouse for cell material is approximately 50 mg/kg.

ASM-1 medium composition (mg/l) is as follows:

NaNO<sub>3</sub> = 170; K<sub>2</sub>HPO<sub>4</sub> = 17.4; Na<sub>2</sub>HPO<sub>4</sub> = 14.2; MgCl<sub>2</sub> = 19.02;  
MgSO<sub>4</sub> = 24.08; CaCl<sub>2</sub> = 22.2; NaEDTA = 6.64; FeCl<sub>3</sub> = 0.65;  
H<sub>3</sub>BO<sub>3</sub> = 2.47; MnCl<sub>2</sub> = 0.87; ZnCl<sub>2</sub> = 0.44; CoCl<sub>2</sub> = 0.01;  
CuCl<sub>2</sub> = 0.0001; Tricine (N-tris[Hydroxy methyl] methyl glycine) =  
26.9; pH 8.5 before autoclaving (Carmichael, 1985).

7. Analytical Procedures:

- a. Biological - Assay for toxicity (of field bloom, cultured cells or toxin extracts) is by mouse bioassay (intraperitoneal injection) using the standard measure, mouse unit (M.U.). M.U. is defined as the amount of toxin required to kill a 20 ± 2 g mouse in 15 minutes.
- b. Extraction and Purification - The extraction of the toxins is shown below. Most of the chlorophyll pigments are precipitated by the acidified ethanol and removed by the ultrafiltration membrane and disposable mini-column Sep-pak C<sub>18</sub>.

Freeze dried cells (1-2 g)

1 M acetic acid in 20% ethanol  
Sonicate 5 min

Centrifuge 4750 G one hour\*

Reduce supernatant volume (5 ml)

Centrifuge 4750 G 5 min

Supernatant

Ultrafiltration

1. Amicon YM-10 Membrane
2. Millipore 0.45 micron filter

Gel filtration  
Sephadex (G-15)

Sep-pak C<sub>18</sub> (sample prep)

Semi preparative HPLC  
CN column - 10 mM ammonium acetate  
1 mM acetic acid

\*The pellet was re-extracted 3X by repeating the previous two steps.



8. Biosynthesis - (N.A.)

9. Toxicology:

- a. Sensitive Hosts - Anatoxin-a causes death by respiratory arrest. Any animal having acetylcholine as the chemical transmitter for muscle conduction can be affected (Carmichael et al., 1975; Carmichael et al., 1979).
- b. The LD<sub>50</sub> (intraperitoneal mouse) for antx-a is about 200 ug/mg (Carmichael et al., 1975; Carmichael et al., 1979). Oral toxicities vary with the animal species but range from 120 mg/kg in goldfish to about 2000 mg/kg in mice for the lyophilized cells. Oral toxicity data for the purified toxin is not available.
- c. Based on laboratory observations in rats and mice the acute (lethal) signs of poisoning include muscle tremors (1-2 min); muscle fasciculations (2-4 min); convulsions (3-5 min); respiratory arrest (5-9 min).
- d. Gross pathology is absent since the toxin is a neurotoxin.
- e. Molecular Basis of Action - Antx-a is a post-synaptic depolarizing neuromuscular blocking agent (Carmichael et al., 1975; Carmichael et al., 1979). It reacts specifically with the acetylcholine receptor. Compared to acetylcholine, succinylcholine, carbachol, decamethonium and nicotine, it is the most potent nicotinic agonist known (Spivak et al., 1980). It may also have some presynaptic activity leading to an increase in miniature end plate current (Biggs and Dryden, 1977).
- f. Synergism Data - (N.A.)
- g. Structure: Function Studies - (N.A.)

10. Detoxification/Decontamination: No work has been done to date.

11. Purified antx-a is a white crystalline powder, mildly hygroscopic and electrostatic. It is stable in aqueous acidic environments at room temperature and above for at least 10 years. Spills should be cleaned up with aqueous/detergent washes at alkaline pH.

12. References:

- 1. Bates, H.A. and H. Rapoport, Synthesis of anatoxin-a via intramolecular cyclization of iminium salts. J. Am. Chem. Soc. 101: 1259-1265, 1979.

2. Biggs, D.F. and W.F. Dryden, Action of anatoxin-I at the neuromuscular junction. Proc. West. Pharmacol. Soc. 20: 461-466, 1977.
3. Campbell, H.F., O.E. Edwards, J.W. Elder, and R.J. Kolt, Total synthesis of DL-anatoxin-a and DL-isoanatoxin-a. Pol. J. Chem. 53: 27-36, 1979.
4. Campbell, H.F., O.E. Edwards, and R.J. Kolt, Synthesis of noranatoxin-A and anatoxin-A. Can. J. Chem. 55: 1372-1379, 1977.
5. Carmichael, W.W., Isolation, culture and toxicity testing of toxic freshwater cyanobacteria (blue-green algae). Proc. ISHC Symposium, Leningrad, USSR, Sept. 1984. Gordon & Breach, New York (In Press), 1985.
6. Carmichael, W.W., D.F. Biggs, and P.R. Gorham, Toxicology and pharmacological action of Anabaena flos-aquae toxin. Science 187: 542-544, 1975.
7. Carmichael, W.W., D.F. Biggs, and M.A. Petersen, Pharmacology of anatoxin-a, produced by the freshwater cyanophyte Anabaena flos-aquae NRC-44-1. Toxicol. 17: 229-236, 1979.
8. Carmichael, W.W. and P.R. Gorham, Anatoxins from clones of Anabaena flos-aquae isolated from lakes of western Canada. Mitt. Int. Verein. Limnol. 21: 285-295, 1978.
9. Devlin, J.P., O.E. Edwards, P.R. Gorham, N.R. Hunter, R.K. Pike, and B. Stavric, Anatoxin-a, a toxic alkaloid from Anabaena flos-aquae NRC-44h. Can. J. Chem. 55: 1367-1371, 1977.
10. Gorham, P.R., J. McLachlan, U.T. Hammer, and W.K. Kim, Isolation and culture of toxic strains of Anabaena flos-aquae (Lyngb.) de Breb. Verh. Int. Verein. Limnol. 15: 796-804, 1964.
11. Huber, C.S., The crystal structure and absolute configuration of 2,9-diacetyl-9-azabicyclo(4,2,1)non-2,3-ene. Acta Crystallograph. Vol. B 28: 2577-2582, 1972.
12. Spivak, C.E., B. Witkop, and E.X. Albuquerque, Anatoxin-a: a novel, potent agonist at the nicotinic receptor. Mol. Pharmacol. 18: 384-394, 1980.

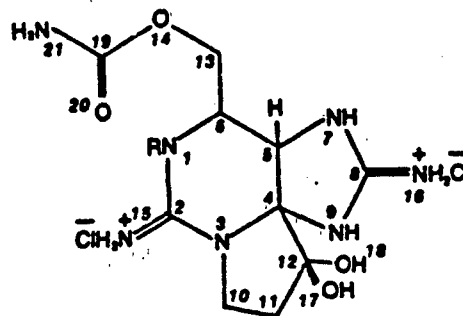
## APHANTOXIN

1. Name: Aphantoxin(s) (Aphtx) synonyms - saxitoxin (Aphtx-II); neosaxitoxin (Aphtx-I); paralytic shellfish poisons.

Ref: (Alam and Euler, 1981; Alam et al., 1973; Alam et al., 1978; Carmichael and Mahmood, 1984; Ikawa et al., 1982, Ikawa et al., 1981; Mahmood and Carmichael, 1985; Sasner et al., 1981).

2. Structure:

R=H (Aphtx-II) - Saxitoxin  
R=OH (Aphtx-I) - Neosaxitoxin



Ref: (Mahmood and Carmichael, 1985; Shimizu et al., 1984).

3. Producing Biological Forms:

Aphanizomenon flos-aquae L. Ralfs. Strain number NH-1 and NH-5. A freshwater filamentous blue-green alga (cyanobacteria) in the family Nostocaceae.

Ref: (Carmichael and Mahmood, 1984; Mahmood and Carmichael, 1985)

4. Physico-chemical Properties:

- a. Elemental - Aphtx-I (neosaxitoxin) =  $C_{10}H_{17}N_7O_5$   
Aphtx-II (saxitoxin) =  $C_{10}H_{17}N_7O_4$

Ref: (Mahmood and Carmichael, 1985; Shimizu et al., 1984)

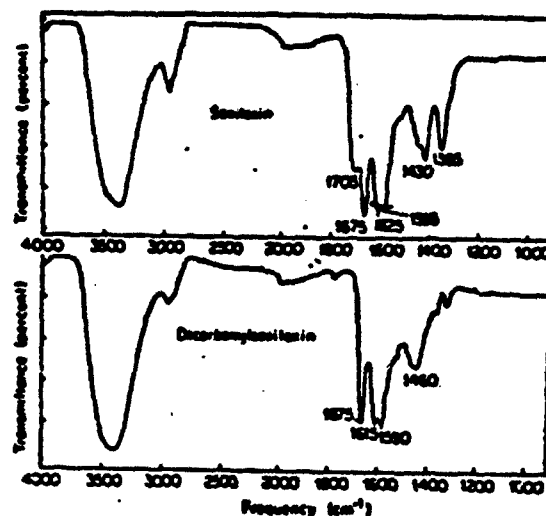
- b. M.W. - Aphtx-I (neosaxitoxin) = 352 (no HCl)  
Aphtx-II (saxitoxin) = 336 (no HCl)

- c. M.P. - (N.A.)

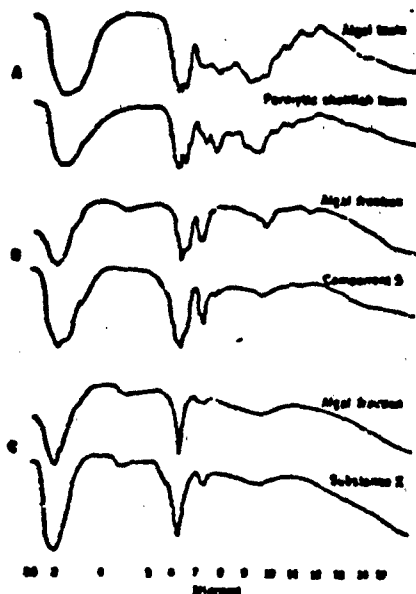
- d. Optical Rotation - (N.A.)

- e. Spectroscopic - U.V. - (N.A.)

f. Spectroscopic - I.R.

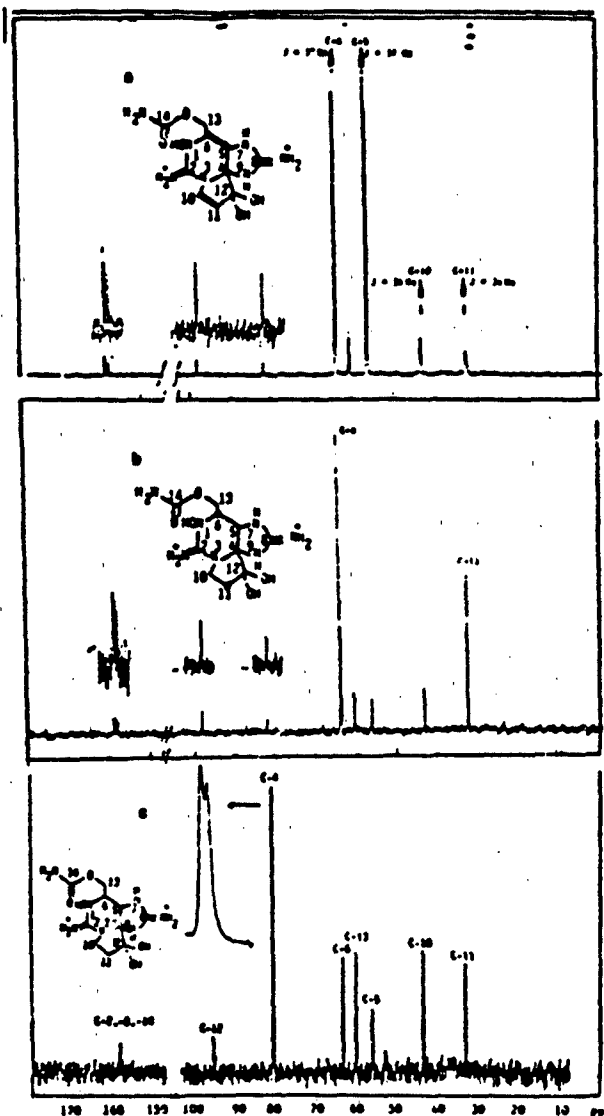


I.R. spectra of saxitoxin and decarbamylsaxitoxin in 99.8% D<sub>2</sub>O in 0.1 mm CaF<sub>2</sub> cells (Ghazarossian et al., 1976).



Infrared spectra of the major fractions of blue-green algal toxin and paralytic shellfish toxin. (A) The most toxic fraction from paper chromatography,  $R_F=0.32$  (B) Blue-green algal fraction from alumina column compared to shellfish toxin fraction 5, redrawn from Casselman et al., (Alam and Euler, 1981). (C) Toxic blue-green algal fraction,  $R_F=0.57$ , compared to compound X from shellfish toxin (Jackim and Gentile, 1968).

g. Spectroscopic - NMR -



$^{13}\text{C}$  NMR spectra of neosaxitoxin isolated from Aphanizomenon flos-aquae fed with (a) [1,2- $^{13}\text{C}$ ]acetate, (b) 2- $^{13}\text{C}$ ]acetate, and (c) [2- $^{13}\text{C}$ , 2- $^{15}\text{N}$ ]ornithine (Shimizu et al., 1984).

h. Spectroscopic - Mass Spec. - (N.A.)

i. Spectroscopic - Circular Dichroism - (N.A.)

j. Solubility - Soluble in water, methanol, ethanol.  
Insoluble in acetone, ether and chloroform.

Ref: (Mahmood and Carmichael, 1985; Sawyer et al., 1968)

k. X-ray Crystallography - (N.A.)

1. Crystal Description - (N.A.)
- m. Specific Gravity - (N.A.)
- n. Misc. Properties - Acid, light and heat stable; alkaline labile; readily dialyzable.  
Ref: (Mahmood and Carmichael, 1985; Sawyer et al., 1968)

## 5. Chemistry:

- a. Derivatives - Aphtx-I is a precursor to Aphtx-II. Both toxins would be derived from a hydropurine ring structure that involves about 12 toxins referred to as paralytic shellfish poisons.  
Ref: (Carmichael and Mahmood, 1984)

- b. Chemical Synthesis - (N.A.)

## 6. Production:

Aph. flos-aquae NH-1 or NH-5 is batch cultured (22-24°C) in BG-11 mineral medium (Carmichael, 1982; Carmichael and Mahmood, 1984; Mahmood and Carmichael, 1985) using continuous lighting from cool white fluorescent and Durotest® Vitalight bulbs. Light intensity is 70-90 uEin/m<sup>2</sup>/sec of photosynthetically active radiation as measured at the culture surface by a Lambda LI-80 meter and quantum sensor. Cultures are harvested at the stationary growth phase (12-15 days) when the cell counts were about 4-6 x 10<sup>6</sup> cells ml<sup>-1</sup>. Harvesting is done using a Millipore Pellicon cell concentrator. Cells are then lyophilized in a Labconco freeze dryer 18. Lyophilized material is stored at -80°C until used for toxin extraction.

An average of 72 mg/liter lyophilized cells is produced from batch cultured Aph. flos-aquae NH-5. The LD<sub>50</sub> (i.p.) mouse is approximately 5 mg/kg for the lyophilized cells. No attempt has been made to maximize culture yield by the use of increased light intensity of carbon dioxide (CO<sub>2</sub>) enrichment. All cultures reach a stationary phase of growth after 12-15 days when inoculated at 5% v/v using log phase inoculation cultures.

BG-11 medium composition is listed as #616 in the American Type Culture Collection catalogue of strains I (1982). Its composition (g/liter) is as follows: NaNO<sub>3</sub> = 1.5; K<sub>2</sub>HPO<sub>4</sub> = 0.04; MgSO<sub>4</sub> · 7H<sub>2</sub>O = 0.075; CaCl<sub>2</sub> · 2H<sub>2</sub>O = 0.36; citric acid = 0.006; ferric ammonium citrate = 0.006; EDTA (Disodium salt) = 0.001; Na<sub>2</sub>CO<sub>3</sub> = 0.02; 1 ml/liter of trace metal mix A5. Trace metal mix A5 has a composition of: H<sub>3</sub>BO<sub>3</sub> = 2.86 g; MnCl<sub>2</sub> · 4H<sub>2</sub>O = 1.81 g; ZnSO<sub>4</sub> = 0.222 g; Na<sub>2</sub>MoO<sub>4</sub> · 2H<sub>2</sub>O = 0.39 g; CuSO<sub>4</sub> · 5H<sub>2</sub>O = 0.079 g; Co(NO<sub>3</sub>)<sub>2</sub> · 6H<sub>2</sub>O = 49.4 mg; distilled water = 1.0 water = 1.0 liter. The pH should be about 7.1 after sterilization.

## 7. Analytical Procedures:

a. Biological - Assay for toxicity (of field bloom, cultured cells or toxin extracts) is by mouse bioassay (intraperitoneal injection) using the standard measure, mouse unit (M.U.). M.U. is defined as the amount of toxin required to kill a  $20 \pm 2$  g mouse in 15 minutes. Correlation between mouse units and survival times are obtained from Sommers table in the American Organization of Analytical Chemists (1975).

b. Extraction and Purification - The extraction of the toxins is shown below (Mahmood and Carmichael, 1985). Most of the chlorophyll pigments are precipitated by the 80% acidified ethanol and removed by the ultrafiltration membrane and disposable mini-column Sep-pak C<sub>18</sub>.

Freeze dried cells (1-2 g)

Acidified water pH 3.0-4.0 (50-100 ml)  
Sonicate 5 min

Centrifuge 27000 g 15°C 45-60 min

Supernatant freeze dried\*

Acidified ethanol (80% pH 4.0) 20-40 ml

Centrifuge 27000 g 15°C 15 min

Supernatant, dried, reconstituted\*\*  
in 0.1 M acetic acid

Ultrafiltration, Diaflo YM-5 membrane

Filtrate (2-3 ml)

Gel filtration (Bio-Gel P-2)

Toxic fractions through Sep-pak C<sub>18</sub>

Ion-exchange chromatography CM-Sephadex G-25

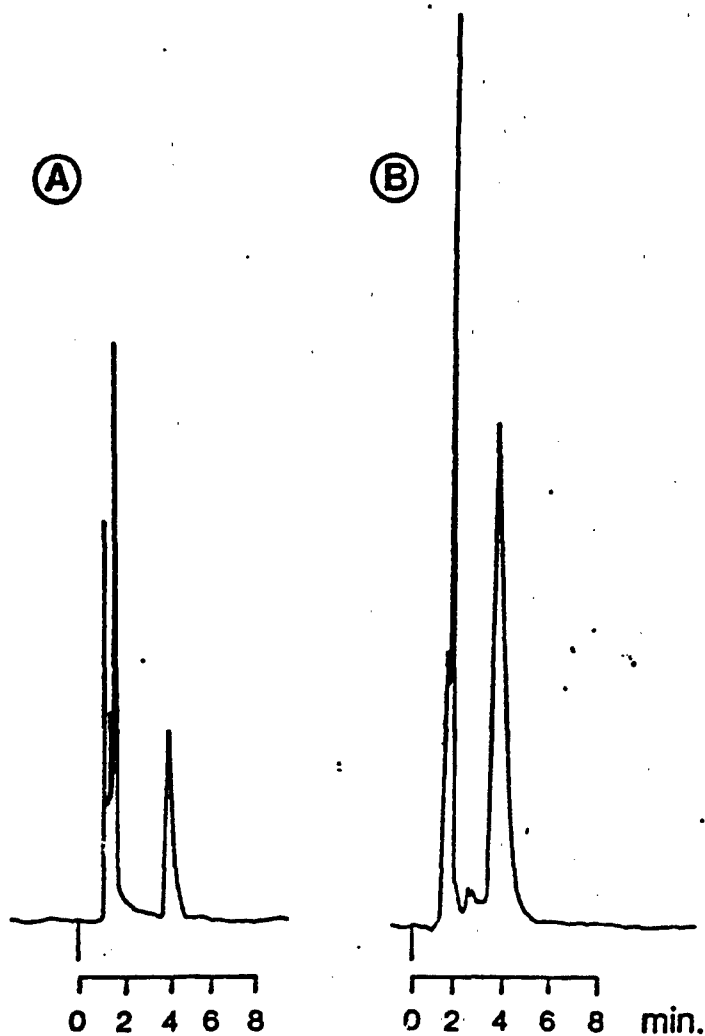
High performance liquid chromatography

\*The pellet was re-extracted 3X by repeating the previous two steps.

\*\*The pellet was dissolved in 80% ethanol and centrifuged. The procedure was repeated 2-3X.

HPLC - Aphantoxin I - Analytical High Performance Liquid Chromatography of Aphtx I and Neo-saxitoxin:  
Ref: (Mahmood and Carmichael, 1985)

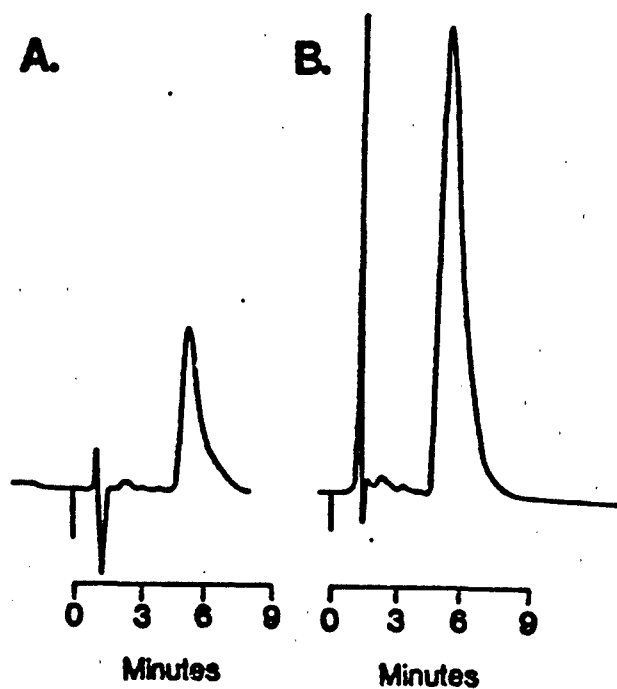
- a) Neo-saxitoxin 18.0 ug; retention time 3 min 40 sec
- b) Neo-saxitoxin (18.0 ug) + Aphtx I (40.0 ug); retention time 3 min 50 sec. Column - Altex CN (4.6 x 150 mm); mobile phase - 10 mM  $\text{NH}_4\text{H}_2\text{PO}_4$ : methanol (60:40); flow rate - 1.0 ml/min; detection - 0.5 AUFS, 220 nm.





HPLC - Aphantoxin II - Analytical High Performance Liquid  
Chromatography of Aphtx II and Saxitoxin:  
Ref: (Mahmood and Carmichael, 1985)

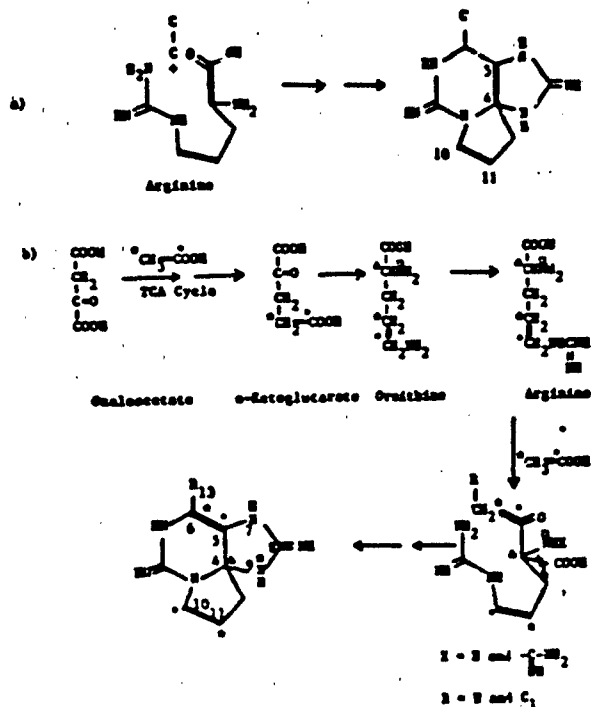
- a) Saxitoxin 2.9 ug; retention time 6 min
- b) Saxitoxin (5.8 ug) + Aphtx II (4.0 ug); retention time  
5 min 59 sec. Column - Altex CN  
(4.6 x 150 mm); mobile phase - 10 mM  
 $\text{NH}_4\text{H}_2\text{PO}_4$ : methanol (60:40); flow rate - 1.0 ml/min  
detection - 0.1 AUFS, 240 nm.



## 8. Biosynthesis:

Feeding of  $1,2\text{-}^{13}\text{C}$  acetate;  $2\text{-}^{13}\text{C}$  acetate and  $[2\text{-}^{13}\text{C}, 2\text{-}^{15}\text{N}]\text{Ornithine}$  (the biosynthetic precursor of arginine) has been used by Shimizu et al., 1984 to study carbon incorporation into the hydropurine ring (Scheme I below). The key finding of the study led to the proposal of a new pathway for biosynthesis of neosaxitoxin (Aphtx-I). The key step in this synthesis is the claisen-type condensation of an acetate unit or its derivative to the amino group bearing alpha-carbon or arginine or an equivalent and a subsequent loss of the carboxyl carbon and imidazole ring formation on the adjacent carbonyl carbon. In this scheme the C-2 and alpha-amino group of arginine or its precursors are incorporated into the toxin molecule in an intact form.

Scheme I



## 9. Toxicology:

- Sensitive Hosts** - As with saxitoxin/neosaxitoxin, respiratory depression is the most prominent action of Aphtx-I and II. Death is due to the respiratory arrest. Since the toxin affects Na channel conduction any organism depending on this type of ion movement for neuromuscular activity can be affected (Adelman et al., 1982).
- LD<sub>50</sub>** (intraperitoneal mouse) for Aphtx-I and II is about 10 ug/kg (the same as saxitoxin/neosaxitoxin) (Mahmood and Carmichael, 1985). Some studies have been done on

fish to demonstrate toxicity (Sawyer et al., 1968) but data on toxicity to other organisms is lacking.

- c. Gross Symptomology for Aphtx I and II is based on observations in laboratory test animals, especially mice. Acute (at the LD<sub>100</sub>) symptoms in mice include muscle tremors (1-2 min); muscle fasciculations (2-4 min); convulsions (3-5 min); respiratory arrest (5-7 min).
  - d. There is no obvious gross pathology since the toxin is a neurotoxin.
  - e. Molecular Basis of Action - Aphantoxin I and II is a specific inhibitor of voltage dependent Na<sup>+</sup> channels (Adelman et al., 1982).
  - f. Synergism Data - (N.A.)
  - g. Structure: Function Studies - (N.A.)
10. Detoxification/Decontamination: No work has been done on detox/decon of Aphtx I or II. Since they are the same as neosaxitoxin and saxitoxin they can be handled in the same way that they are.
11. Aphtx-I-II when purified and lyophilized are white crystalline powders, which are mildly hygroscopic and electrostatic. They are stable in aqueous acidic environments at room temperature and above for periods of at least 1 year. Spills should be cleaned up with aqueous/detergent washes at alkaline pH.
12. References:
- Adelman, W.J., Jr., J.F. Fohlmeister, J.J. Sasner, Jr., and M. Ikawa, Sodium channels blocked by aphantoxin obtained from the blue-green alga, Aphanizomenon flos-aquae. Toxicon 20: 513-516, 1982.
- Alam, M. and K.L. Euler. Chemical studies on toxins from the blue-green alga Aphanizomenon flos-aquae. In: Carmichael, W.W. (ed.) The Water Environment: Algal Toxins and Health. Plenum Press, New York, p. 405-414, 1981.
- Alam, M., M. Ikawa, J.J. Sasner, Jr., and P.J. Sawyer, Purification of Aphanizomenon flos-aquae toxin and its chemical and physiological properties. Toxicon 11: 65-72, 1973.
- Alam, M., Y. Shimizu, M. Ikawa, and J.J. Sasner, Jr., Reinvestigation of the toxins from the blue-green alga Aphanizomenon flos-aquae by a high performance chromatographic method. J. Environ. Sci. Health A11: 493-499, 1978.

Association of Official Analytical Chemists. Official methods of analysis, 12th Ed., revised. AODC, Washington, D.C., 1975.

Carmichael, W.W., Chemical and toxicological studies of the toxic freshwater cyanobacteria Microcystis aeruginosa, Anabaena flos-aquae and Aphanizomenon flos-aquae. S. Afr. J. Sci. 78: 367-372, 1982.

Carmichael, W.W. and N.A. Mahmood. Toxins from freshwater cyanobacteria (blue-green algae). In: Ragelis, E.P. (ed.) Seafood Toxins. ACS Symposium Series 262, American Chemical Society, Washington, D.C., p. 377-389, 1984.

Ghazarossian, V.E., E.J. Schantz, H.K. Schnoes, and F.M. Strong, A biologically active acid hydrolysis product of saxitoxin. Biochem. Biophys. Res. Comm. 68: 776-780, 1976.

Ikawa, M., K. Wegener, T.L. Foxall, and J.J. Sasner, Jr., Comparison of the toxins of the blue-green alga Aphanizomenon flos-aquae with the Gonyaulax toxins. Toxicon 20: 747-752, 1982.

Ikawa, M., K. Wegener, T.L. Foxall, J.J. Sasner, Jr., P.W. Carter, and N.H. Shoptaugh. Studies on the fluorometric determination of the toxins of the blue-green alga Aphanizomenon flos-aquae. In: Carmichael, W.W. (ed.) The Water Environment: Algal Toxins and Health. Plenum Press, New York, p. 415-425, 1981.

Jackim, E. and J. Gentile, Toxins of blue-green alga: similarity to saxitoxin. Science 162: 915-916, 1968.

Mahmood, N.A. and W.W. Carmichael, Paralytic shellfish poisons produced by the freshwater cyanobacterium Aphanizomenon flos-aquae NH-5. Toxicon (In Press), 1985.

Sasner, J.J., Jr., M. Ikawa, and T.L. Foxall. Studies on Aphanizomenon and Microcystis toxins. In: Ragelis, E.P. (ed.) Seafood Toxins. ACS Symposium Series 262, American Chemical Society, Washington, D.C., p. 391-406, 1984.

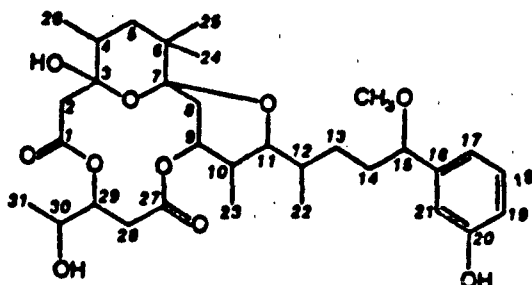
Sasner, J.J., Jr., M. Ikawa, T.L. Foxall, and W.H. Watson. Studies on aphantoxin from Aphanizomenon flos-aquae in New Hampshire. In: Carmichael, W.W. (ed.) The Water Environment: Algal Toxins and Health. Plenum Press, New York, p. 389-402, 1981.

Sawyer, P.J., J.H. Gentile, and J.J. Sasner, Jr., Demonstration of a toxin from Aphanizomenon flos-aquae (L.) Ralfs. Can. J. Microbiol. 14: 1199-1204, 1968.

Shimizu, Y., M. Norte, A. Hori, A. Genenah, and M. Kobayashi, Biosynthesis of saxitoxin analogues: the unexpected pathway. J. Am. Chem. Soc. 106: 6433-6434, 1984.

APLYSIATOXIN/DEBROMOAPLYSIATOXIN/OSCILLATOXIN A

1. Name: Aplysiatoxin/Debromoaplysiatoxin/Oscillatoxin A  
Ref: (Moore et al., 1984; Mynderse et al., 1977)
2. Structure:



Debromoaplysiatoxin

Aplysiatoxin: Bromine at C<sub>17</sub>

Oscillatoxin A from Oscillatoria has an H group at the C<sub>31</sub> position Instead of a methyl group.

Ref: (Moore et al., 1984)  
Registry No.: Aplysiatoxin = 52659-57-1  
Debromoaplysiatoxin = 52423-2B-6  
Oscillatoxin A = 66671-95-2

3. Producing Biological Forms:

Lyngbya majuscula Gomont, Oahu, Hawaii, Okinawa, and Marshall Is.: Schizothrix calcicola and Oscillatoria nigroviridis, Gilbert Islands and Marshall Is. All are filamentous blue-green algae (cyanobacteria) in the family Oscillatoriaceae.

Ref: (Moore et al., 1984; Mynderse et al., 1977)

4. Physico-chemical Properties:

a. Elemental - Aplysiatoxin = C<sub>32</sub>H<sub>49</sub>O<sub>10</sub>Br  
Debromoaplysiatoxin = C<sub>32</sub>H<sub>50</sub>O<sub>10</sub>  
Oscillatoxin A = C<sub>31</sub>H<sub>48</sub>O<sub>10</sub>

b. M.W. - Aplysiatoxin = 626  
Debromoaplysiatoxin = 592  
Oscillatoxin A = 478  
Ref: (Mynderse et al., 1977)

c. M.P. - Debromoaplysiatoxin = 105.2 - 107.0°C  
Ref: (Moore, 1984)

d. Optical Rotation - Debruoaplysiatoxin = [  $\alpha$  ]<sup>25</sup><sub>D</sub>+60.6°  
(EtOH, C 0.66) by crystallization from diethyl ether/n-  
pentance(1:1) at -10°C and recrystallization from aqueous  
methanol.

Ref: (Moore et al., 1984)

e. Spectroscopic - U.V. - (N.A.)

f. Spectroscopic - I.R. - (N.A.)

APLYSIATOXIN/DEBROMOAPLYSIATOXIN/OSCILLATOXIN-A

g. Spectroscopic - NMR -

H NMR Data for Aplysiatoxins and Oscillatoxin A in Acetone-d.

	1 <sup>a</sup>	2 <sup>b</sup>	3 <sup>b</sup>
H-2	2.628 (d)	2.53	2.53
H-2	2.744 (d)	2.77	2.74
OH on C(3)	4.292 (d)	4.30	4.35
H-4	1.88 (m)	1.86	1.84
H-5 ax	1.625 (t)	1.63	1.62
H-5 eq	1.064 (dd)	1.06	1.06
H-8 ax	1.724 (dd)	1.71	1.72
H-8 eq	2.699 (dd)	2.70	2.72
H-9	5.241 (m)	5.24	5.21
H-10	1.742 (m)	1.70	1.70
H-11	3.941 (dd)	3.94	3.92
H-12	1.54 (m)	1.53	1.53
H-13	1.50 (m)	1.4	1.40
H-13	1.43 (m)	1.3	1.32
H-14	2.00 (m)	1.98	1.95
H-14	1.6 (m)	1.60	1.60
H-15	4.485 (t)	4.02	3.99
H-17		6.86	6.84
H-18	7.32B (d)	7.16	7.13
H-19	6.702 (dd)	6.74	6.72
OH on C(20) <sup>c</sup>		8.21	8.25
H-21	7.106 (d)	6.95	6.93
3H-22	0.826 (d)	0.80	0.80
3H-23	0.740 (d)	0.72	0.73
3H-24	0.850 (s)	0.82	0.81
3H-25	0.861 (s)	0.85	0.84
3H-26	0.860 (d)	0.88	0.86
H-28	2.907 (m)	2.93	2.93
H-28	2.895 (m)	2.91	2.96
H-29	5.208 (m)	5.25	5.22
H-30	3.999 (dq)	4.07	3.67, 3.69
OH on C(30) <sup>d</sup>		4.23	4.14
3H-31	1.111 (d)	1.14	
OCH <sub>3</sub>	3.194 (s)	3.19	3.17

<sup>a</sup> J (Hz) for 1: 2,2'=-12.6; 3(OH), 4=2.0; 4,5(ax)=13.0; 4,5(eq)=3.7; 4,26=6.4; 5,5=-13.3; 8,8=-14.6 8(ax),9=3.6; 8(eq),9=3.0; 9,10=3.3; 10,11=10.7; 10,23=6.9; 11,12=2.0; 12,22=6.6; 14,15=14', 15=6.5; 18,19=8.5; 19,21=3.0; 28,28'=-18.1; 28,29=11.6; 28', 29=2.3; 29,30=4.1; 30,31=6.5.

<sup>b</sup> J (Hz) for 2 and 3: see Moore et al., 1984.

<sup>c</sup> Broad singlet

<sup>d</sup> OH signals for 1 not observed due to the presence of a small amount of D<sub>2</sub>O in the solvent. Ref: (Moore et al., 1984)

- h. Spectroscopic - Mass Spec. - Debromoaplysiatoxin shows parent ion peak at M.W. 592 + strong peak at 574 for loss of water from the molecular ion.  
Ref: (Mynderse et al., 1977)
- i. Spectroscopic - Circular Dichroism - Debromoaplysiatoxin shows a positive Cotton effect with  $[\theta]_{269} + 902^\circ$  and  $[\theta]_{286} + 1031^\circ$  (EtOH). Oscillatoxin A =  $[\theta]_{269} + 1022^\circ$  and  $[\theta]_{282} + 1255^\circ$  (EtOH).  
Ref: (Moore et al., 1984)
- j. Solubility - soluble in methylene chloride, isopropanol and dichloromethane; insoluble in water.  
Ref: (Moore et al., 1984)
- k. X-ray Crystallography - 19,21-dibromoplysiatoxin crystals (grown in methanol-water solutions at 20-25°C) gave triclinic symmetry with accurated lattice constants of  $a=9.859(5) \text{ \AA}$ ,  $b=9.929(5) \text{ \AA}$ ,  $c=11.351(7) \text{ \AA}$ ,  $\alpha=101.132(44)^\circ$ ,  $\beta=112.072(41)^\circ$ , and  $\gamma=74.941(39)^\circ$ .  
Ref: (Moore et al., 1984)
- l. Crystal Description - 19,21-dibromoplysiatoxin-rectangular 0.6 x 0.5 x 0.3 mm  
Ref: (Moore et al., 1984)
- m. Specific Gravity - (N.A.)
- n. Misc. Properties - (N.A.)
5. Chemistry:
- a. Derivatives - Several brominated derivatives can be obtained by bromine treatment of debromoaplysiatoxin in aqueous methanol buffered at pH 6.  
Ref. (Moore et al., 1984)
- b. Chemical Synthesis - (N.A.)
6. Production:

All isolations to date of aplysiatoxin, debromoaplysiatoxin and oscillatoxin A have been made from natural collections of the algal mats. These include areas of Hawaii and the South Pacific. Laboratory culture of Lyngbya majuscula for aplysiatoxin or debromoaplysiatoxin Schizothrix calcicola or Oscillatoria nigroviridis for oscillation A, is made difficult by the presence of other algae attached to the filaments. A suitable culture medium is also not available.  
Ref: (Moore et al., 1984; Mynderse et al., 1977).



## APLYSIATOXIN/DERROMOAPLYSIATOXIN/OSILLATOXIN A

### 7. Analytical Procedures:

- a. Biological - Although the mouse bioassay has been used to test toxicity other assays are more commonly used. These include:
- 1) Inflammatory activity by the mouse ear test.
  - 2) Inflammatory activity on the skin of hairless mice (activity observed at about 5 parts in 10,000,000),  
Ref: (Soloman and Stoughton, 1978)
  - 3) Activity against P-388 lymphocytic mouse leukemia cells  
Ref: (Mynderse et al., 1977)
  - 4) Tumor promotion in mouse skin
  - 5) Adhesion of human promyelocytic leukemia cells (HL-60)  
Ref: (Fujiki et al., 1983; Moore, 1984; Mynderse et al., Soloman and Stoughton, 1978)
- b. Extraction and Purification - The extraction and purification of aplysiatoxin, debromoaplysiatoxin and oscillatoxin A is as follows:

#### Aplysiatoxin and Debromoaplysiatoxin:

- 1) Lyophilized cells extracted with a 1:1 mixture of methylene chloride and isopropanol
- 2) Partition with 1:1 hexane/10% aqueous methanol (discard hexane layer).
- 3) Adjust aqueous methanol layer to 35% water.
- 4) Extract 35% aqueous methanol with 500 ml of methylene chloride
- 5) Gel filtration of resulting gum on Sephadex LH-20 (2.5 cm x 80 cm) with 1:1 methylene chloride isopropanol.
- 6) Biologically active fraction (mouse ear irritation) purified by reverse phase liquid chromatography (acetonitrile: water 7:3) on a C-18 column (Partisil - 10 ODS) - followed by liquid chromatography on a CN Bondapak with 8% ethyl acetate in water.  
Ref: (Moore et al., 1984)

#### Oscillatoxin A:

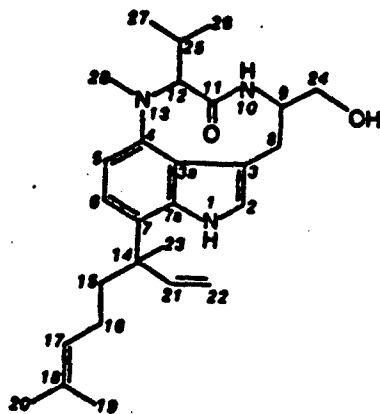
- 1) Extract cells of Schizothrix calcicola/Oscillatoria nigroviridis with chloroform: methanol (1:1).

- 2) Chromatograph extract on a 20 x 3.7 cm column of Florisil with 75% hexane/CHCl<sub>3</sub>, 25% CHCl<sub>3</sub> and 50% hexane/CHCl<sub>3</sub>, 50% CHCl<sub>3</sub> gave two toxic fractions (A+B).
  - 3) Fraction A was further purified by gel filtration on Sephadex LH-20 with 1:1 MeOH:CHCl<sub>3</sub> - this yielded debromoaplysia toxin.
  - 4) Fraction B was purified by gel filtration of Sephadex LH-20 (2.5 x 1.9 cm column) with 1:1 MeOH:CHCl<sub>3</sub>. This was followed by HPLC on Porasil A with 30% CH<sub>3</sub>CN in CHCl<sub>3</sub>. This material was oscillatoxin A.
8. Biosynthesis: (N.A.)
9. Toxicology:
- The brominated phenolic based toxins aplysiatoxin, debromoaplysiatoxin and oscillatoxin A are all potent contact irritants producing erythema, blisters and necrosis in humans and laboratory test animals, within minutes to hours and lasting several days. They are also potent tumor promoters in vivo, comparable in activity to 12-o-tetradecanoylphorbol-13-acetate (TPA) from Croton Oil. The toxins have been encountered when people are swimming in shallow areas around parts of Oahu and Okinawa. It is possible that Lyngbya majuscula, Schizothrix calcicola and Oscillatoria nigroviridis could be ingested by humans as epiphytes attached to edible seaweeds such as Asparagopsis taxiformis (Hawaiian name: Limu Kohu) and Laurencia nidifica (Hawaiian name: Limumane'ono'o).
- Ref: (Fujiki et al., 1983; Moore, 1984; Solomon and Stoughton, 1978; Suganuma et al., 1984)
10. Detoxication/Decontamination - (N.A.)
  11. These toxins are all clear oil substances soluble in a variety of apolar organic chemicals. Stability and cleanup procedures of the compounds has not been reported in the literature.
  12. References:
    1. Fujiki, H., M. Suganuma, T. Sugimura, and R.E. Moore. New tumor promoters: dihydroteleocidin B, teleocidin, lyngbyatoxin A, aplysiatoxin, and debromoaplysiatoxin. In: Harris, C.C. (ed.) Human Carcinogenesis. Academic Press, London, p. 303-324, 1983.

2. Moore, R.E. Public health and toxins from marine blue-green algae. In: Ragelis: E.P. (ed.) Seafood Toxins, ACS Symposium Series 262. American Chemical Society, Washington, D.C., p. 369-376, 1984.
3. Moore, R.E., A.J. Blackman, C.E. Cheuk, J.S. Mynderse, G.K. Matsumoto, J. Clardy, R.W. Woodard, and J.C. Craig. Absolute stereochemistries of the aplysiatoxins and oscillatoxin A. J. Org. Chem. 49: 2484-2489, 1984.
4. Mynderse, J.S., R.E. Moore, M. Kashiwagi, and T.R. Norton. Antileukemia activity in the oscillatoriaceae: isolation of debromoaplysiatoxin from Lyngbya. Science 196: 538-540, 1977.
5. Soloman, A.E. and R.B. Stoughton. Dermatitis from purified sea algae toxin (debromoaplysiatoxin). Arch. Dermatol. 114: 1333-1335, 1978.
6. Suganuma, M., H. Fujika, H., T. Tahira, C.E. Cheuk, R.E. Moore, and T. Sugimura. Estimation of tumor promoting activity and structure-function relationships of aplysiatoxins. Carcinogenesis 5: 315-318, 1984.

## LYNGBYATOXIN A

1. Name: Lyngbyatoxin A  
Ref: (Cardellina et al., 1979; Moore, 1981)
2. Structure:



Lyngbyatoxin A

Ref: (Cardellina et al., 1979; Moore, 1981)

3. Producing Biological Forms:

Lyngbya majuscula Gomont. Toxin was initially extracted from natural populations of algae found growing attached to rocks on Kahala Beach, Oahu (Cardellina et al., 1979). L. majuscula is a filamentous blue-green alga (Cyanobacteria) in the family Oscillatoriaceae.

4. Physico-chemical Properties:

- a. Elemental -  $C_{27}H_{39}N_3O_2$   
Ref: (Cardellina et al., 1979)
- b. M.W. - 437  
Ref: (Cardellina et al., 1979; Fujika et al., 1983)
- c. M.P. - (N.A.)
- d. Optical Rotation -  $[\alpha]_D - 171^\circ$  (c 1.8,  $CHCl_3$ )  
Ref: (Cardellina et al., 1979)
- e. Spectroscopic - U.V. (in ethanol) - bands present at 212 ( $\epsilon = 18,200$ ), 231 ( $\epsilon = 26,300$ ), 287 ( $\epsilon = 8,900$ ), and 301 nm ( $\epsilon = 9,300$ ).  
Ref: (Cardellina et al., 1979)
- f. Spectroscopic - I.R. - (N.A.).

# LYNGBYATOXIN A

## g. Spectroscopic - NMR

Table 1. Nuclear Magnetic Resonance Data for Lyngbyatoxin A  
Abbreviations: s = singlet; d = doublet; t = triplet; m = multiplet; br = broad; gem = geminal; and J = coupling constant.

Carbon - 13

Chemical shift*	Assignment**	Proton chemical shift
174.7(s)	11	
148.3(d)	21	6.15(dd, $J_{21,22}=18$ , $J_{21,22}=10$ Hz)
146.2(s)	4	
137.2(s)	7a	
131.3(s)	18	
124.4(d)	17	5.06(br, m)
121.4(s)	3, 3a, or 7	
120.7(d)	2	6.81(br, s)
119.7(d)	6	6.96(d, $J_{5,6}=8$ Hz)
118.4(s)	3, 3a, or 7	
114.1(s)	3, 3a, or 7	
112.1(t)	22	5.30(unresolved dd, $J_{21,22}=18$ Hz) 5.26 (unresolved dd, $J_{21,22}=10$ Hz)
106.1(d)	5	6.44 (d, $J_{5,6}=8$ Hz)
70.6(d)	12	4.33(d, $J_{12,25}=12$ Hz)
64.7(t)	24	3.72(dd, $J_{gem}=-11.5$ , $J_{9,24}=3$ Hz) 3.56(dd, $J_{gem}=-11.5$ , $J_{9,24}=8.5$ Hz)
55.7(d)	9	4.32(br, s)
43.0(s)	14	
38.3(t)	15	1.89(td, $J_{gem}=-12$ , $J_{15,16}=J_{16}=3$ Hz) 1.80(td, $J_{gem}=-12$ , $J_{15,16}=J_{15,16}=3$ Hz)
33.7(t)	8	3.11(dd, $J_{gem}=-17.5$ , $J_{8,9}=2$ Hz) 3.05(dd, $J_{gem}=-17.5$ , $J_{8,9}=3.5$ Hz)
32.9(q)	28	2.87(s)
28.1(d)	25	2.55(m)
25.3(q)	23	1.44(s)
23.6(q)	19	1.47(br, s)
22.6(t)	16	1.93(br, m) 1.70(br, m)
21.5(q)	26	0.89(d, $J_{25,26}=6.5$ Hz)
19.3(q)	27	0.62(d, $J_{25,27}=6.5$ Hz)
17.2(q)	20	1.63(br, s)
	1	8.50(br, s)
	10	7.77(br, s)

Table 1 (continued)

OH on 24	Not observed
-----	
*Relative to CDCL <sub>3</sub> (76.9 ppm) as solvent. **Based on proton single frequency off-resonance decoupling experiments at 90 MHz (carbon-13) and proton spin-spin decoupling experiments at 100 and 360 MHz (proton). J <sub>gem</sub> not determined. Becomes a sharper singlet when irradiated at 6.81 ppm. *Becomes a sharper singlet when irradiated at 4.32 ppm.	

Ref: (Cardellina et al., 1979)

- h. Spectroscopic - Mass. Spec. - established m/e at 437.3019  
Ref: (Cardellina et al., 1979)
- i. Spectroscopic - Circular Dichroism (In methanol) - based on tetrahydrolyngbyatoxin-A-  $[\theta]_{210nm} - 22,700$ ,  $[\theta]_{223} - 47,900$ ,  $[\theta]_{235} - 23,700$ ,  $[\theta]_{239} - 30,200$ ,  $[\theta]_{247} - 25,200$ ,  $[\theta]_{265} - 27,700$ ,  $[\theta]_{301} 0$  (Infection),  $[\theta]_{313} + 7,100$ .  
Ref: (Cardellina et al., 1979)
- j. Solubility - Soluble in hexane, diisopropyl ether, ethanol and dichloromethane. Not soluble in water.  
Ref: (Cardellina et al., 1979)
- k. X-ray Crystallography - (N.A.)
- l. Crystal Description - (N.A.)
- m. Specific Gravity - (N.A.)
- n. Misc. Properties - at room temperature is a tan gummy substance.
5. Chemistry:
- a. Derivatives - Lyngbyatoxin A is the same chemical as teleocidin A which is produced by the filamentous bacterium Streptomyces. Hydrogenation of lyngbyatoxin A does not alter its toxic activity.
- Tetrahydrolyngbyatoxin A is as active as the natural toxin.
- Ref: (Cardellina et al., 1979; Fukiki et al., 1983; Moore, 1981)
- b. Chemical Synthesis - (N.A.)
6. Production:
- All isolations to date of lyngbyatoxin A have been made from natural collections of the algal mats. These include areas of Hawaii (Oahu) and Okinawa. Laboratory culture of Lyngbya mujuscula is made difficult by the presence of other algae attached to the filaments. A suitable culture medium is also not available. The yield of pure toxin from lyophilized natural material is about 0.02%. Ref: (Cardellina et al., 1979).
7. Analytical Procedures:
- a. Biological - although the mouse bioassay has been used

to test toxicity ( $LD_{100} = 0.3 \text{ mg/kg}$  - pure toxin) other assays are more commonly used. These include:

- 1) Skin tumor promotion on mouse skin
  - 2) Induction of ornithine decarboxylase (ODC) in mouse skin
  - 3) Adhesion of human promyelocytic leukemia cells (HL-60).
- Ref: (Fujiki et al., 1984; Fujiki et al., 1983)

b. Extraction and purification of lyngbyatoxin A is as follows:

- 1) Extract lyophilized cells with petroleum ether.
- 2) Reextract cell pellets with dichloromethane ( $\text{CH}_2\text{Cl}_2$ ).
- 3) Apply  $\text{CH}_2\text{Cl}_2$  extract to a Sephadex LH-20 column (180 x 2.5 cm) and elute successively with:  
 $\text{CH}_2\text{Cl}_2$ : hexane (4:1);  $\text{CH}_2\text{Cl}_2$ :  $\text{Me}_2\text{CO}$  (3:2);  
 $\text{CH}_2\text{Cl}_2$ :  $\text{Me}_2\text{CO}$  (1:4). The toxic peak occurs after about 800 ml elution with the  $\text{CH}_2\text{Cl}_2$ :hexane (4:1).
- 4) High performance liquid chromatography (HPLC) the toxic fraction on a micro-Bondapak-CN column, with hexane:diisopropyl ether (1:1).

Ref: (Cardellina et al., 1978; Cardellina et al., 1979)

8. Biosynthesis - (N.A.)

9. Toxicology:

The indole alkaloid lyngbyatoxin A is a potent contact irritant producing inflammation and pustules in humans within minutes to a few hours. Contact with the poison is usually made when people are swimming or bathing and the algal mats become caught on bathing suits. It is also potent skin tumor promoter when tested in the mouse. No information is available on tumor promotion in humans. After initiation in mice with 7,12-dimethylbenz(a)anthracene (DMBA), lyngbyatoxin A at 2.5 ug (twice weekly) will induce skin tumors within 5 weeks.

10. Detoxification/Decontamination - (N.A.)

11. Lyngbyatoxin A is a tan gummy substance soluble in organic solvents such as hexane and petroleum ether. Stability and cleanup procedure of the compound has not been reported in the literature.

Ref: (Cardellina et al., 1979)

12. References:



1. Cardellina, J.H. II, D. Dalletos, F.J. Marner, J.S. Mynderse, and R.E. Moore,  
(-)-Trans-7(S)-methoxytetraides-4-enoic acid and related  
amides from the marine cyanophyte Lyngbya majuscula.  
Phytochem. 17: 2091-2095, 1978.
2. Cardellina, J.H., II, F.J. Marner, and R.E. Moore,  
Seaweed dermatitis: structure of lyngbyatoxin A. Science  
204: 193-195, 1979.
3. Fujiki, H., M. Suganuma, H. Hakii, G. Bartolini, R.E.  
Moore, S. Takayama, and T. Sugimura, A two-stage mouse  
skin carcinogenesis study of lyngbyatoxin A. J. Cancer  
Res. Clin. Oncol. 108: 174-176, 1984.
4. Fujiki, H., M. Suganuma, T. Sugimura, and R.E. Moore. New  
tumor promoters: dihydroteleocidin B, teleocidin,  
lyngbyatoxin-A, aplysiatoxin and debromoaplysiatoxin.  
In: Harris, C.C. (ed.) Human Carcinogenesis. Academic  
Press, London, p. 303-324, 1983.
5. Moore, R.E. Toxins from marine blue-green algae. In:  
Carmichael, W.W. (ed.) The Water Environment: Algal  
Toxins and Health. Plenum Press, New York, p. 15-23,  
1981.

## MICROCYSTIN

1. Name: Microcystin (Fast Death Factor);  
Ref: (Bishop et al., 1959)

Name: Cyanoginosin-LA (Toxin BE-4);  
Ref: (Botes et al., 1984)

Name: Toxin-LR;  
Ref: (Slatkin et al., 1983)

2. Structure:

Cyanoginosin-LA (Toxin BE-4) (Fig. 1) = cyclo(-D-Ala-L-Leu-erythro- $\beta$ -methyl-D-isoAsp-L-Ala-Adda-D-Glu-N-methyldehydro-Ala)

Ref: Botes et al., 1984; Santikarn et al., 1983)

Fig. 1: Proposed general structure for cyclicheptapeptide toxins of Microcystis aeruginosa. Ref: (Botes et al., 1984).

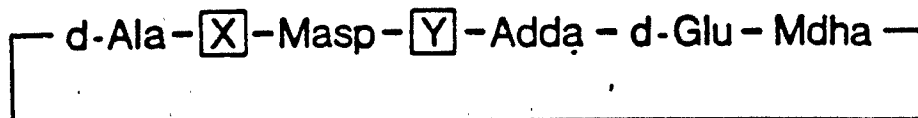
X and Y = variable L-amino acids which can differ between strains.

For Toxin BE-4: X = leucine, Y = alanine.

Masp = B-methylaspartic acid.

Adda = B-amino acid residue of 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid.

Mdha = methyldehydroalanine



Toxin-LR = pentapeptide containing alanine, glutamic acid, B-methylaspartic acid, leucine and arginine. Produced by M. aeruginosa strain 006 and 029 from the University of Orange Free State, Dept. Botany, Republic of South Africa.  
Ref: (Siegelman et al., 1984; Slatkin et al., 1983)

Microcystin = polypeptide containing aspartic acid, glutamic acid, alanine, leucine, serine, valine and ornithine. Produced by M. aeruginosa strain NRC-SS-17, National Research Council, Canada.  
Ref: (Bishop et al., 1959; Runnegar and Falconer, 1981)

Note: Microcystin from M. aeruginosa strain NRC-SS-17 was probably not a pure compound when chemically analyzed. Defini-

tive structure work should refer to toxin BE-4 (cyanoginosa-LA) for the basic structure of this peptide toxin group. The proposed generalized structure for these peptides (Botes et al., 1984) would therefore be a cyclic heptapeptide with three invariant D-amino acids (alanine, glutamic and B-methylaspartic) plus Adda and N-methyldehydroalanine. Two variant L-amino acids are present which in BE-4 are leucine and alanine. Other combinations include leucine-arginine; tyrosine-arginine; methionine-arginine; leucine-tyrosine; alanine-tyrosine or arginine-arginine.

### 3. Producing Biological Forms:

Cyanoginosa-LA is produced by the colonial cyanobacteria (blue-green algae) Microcystis aeruginosa Kutz. emend. Elken. isolated from Witbank Dam, Transvaal, Republic of South Africa, May 1976. Culture strain number is WR-70. Microcystis is in the family Chroococcaceae.

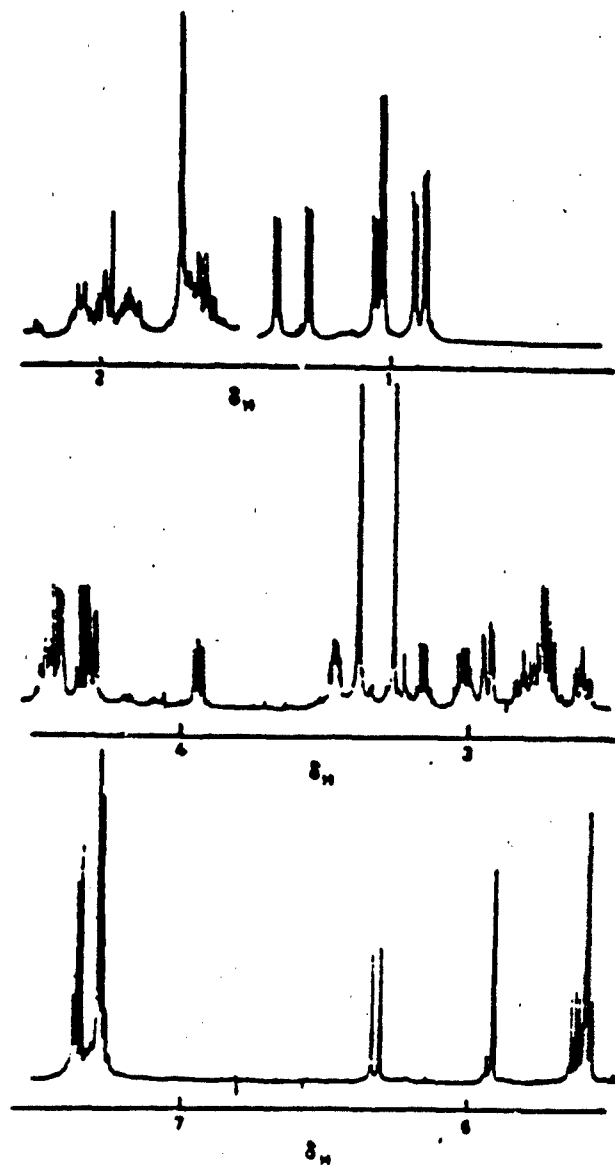
Ref: (Botes et al., 1982; Scott, 1974)

### 4. Physico-chemical Properties:

- a. Elemental - (N.A.)
- b. M.W. - cyanoginosin-LA=909
- c. M.P. - (N.A.)
- d. Optical rotation - (N.A.)
- e. Spectroscopic - U.V. - maximal peak at 238-240 nm.
- f. Spectroscopic - I.R. - (N.A.)

MICROCYSTIN

g. Spectroscopic - NMR -



The 500 M Hz  $^1\text{H}$  n.m.r. spectrum of cyanoginosin-LA in  $\text{D}_2\text{O}$ .

## MICROCYSTIN

## g. Spectroscopic - NMR - continued

The 125 MHz  $^{13}\text{C}$  n.m.r. Data of Cyanoginosin-LA in  $\text{D}_2\text{O}$ 

	Multiplicity	$^1J(\text{CH})/\text{Hz}$	$>^1J(\text{CH})/\text{Hz}$	Assignment
178.10	s			} carbonyls <sup>b</sup>
177.89	s			
176.37	s			
167.39	s			
144.58	s			
140.47	s			} Adda and C of Mdha
138.72	d	160.9		
136.95	d	155		
134.37	s			
130.58	d	159		
129.48	d	163		
129.48	d	163		
127.26	d	162.1	8	
125.70	d	157.3		} C <sub>3</sub> of Mdha
116.43	t	165.1		
87.78	d	151.8		} C-9 of Adda
58.16	q,d	142.1	5	
57.62	d	144		} OCH <sub>3</sub> of D-Ala, L-Ala, Leu, Glx, Masx and C-3 of Adda
56.87	d	ca. 140		
56.19	d	138.9		
54.71	d	137.4		
50.59	d	142.1		
48.81	d	136.3		} C-8 of Adda
44.98	d	136.7		
42.26	d	131		} C-2 of Adda
40.36	t	129		
38.86	q	140.5		} C <sub>β</sub> of Leu
37.87	t	124.0		
36.71	d	123.8		} NCH <sub>3</sub> of Adda
32.72	t	126		
27.70	t	127		} C <sub>β</sub> of Masx
25.35	d	127.4		
23.39	q	126		} C <sub>γ</sub> of Glx
20.99	q	126		
17.38	q	129.2		} C <sub>β</sub> of Glx
17.14	q	130.6		
16.71	q	130		} C <sub>γ</sub> of Leu
15.41	q	125.4		
14.99	q	130.0		} C <sub>δ</sub> of Leu
13.10	q	126		

<sup>a</sup> Relative to  $\text{SiMe}_4$  measured from internal dioxane and corrected by using  $\delta_{\text{C}} = (\text{SiMe}_4) = \delta_{\text{C}} = (\text{dioxane}) + 67.8$  p.p.m.

<sup>b</sup> Not all the carbonyl resonances could be detected under the experimental conditions used. Ref: (Botes et al., 1984)

# MICROCYSTIN

## h. Spectroscopic - Mass Spec. -

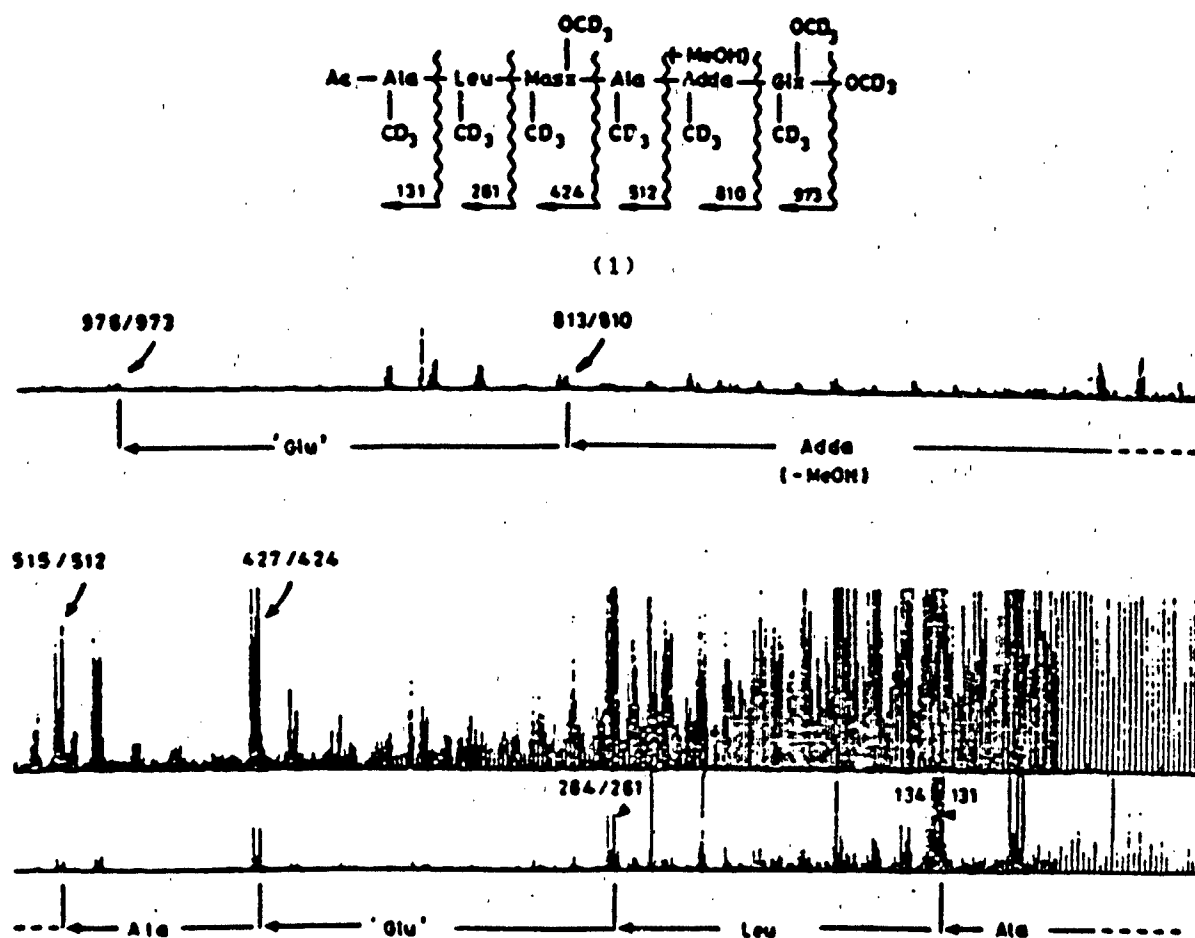


Fig. 2: Electron-impact mass spectrum of the ( $^3\text{H}_\Delta/^3\text{H}_\Delta$ ) acetylated derivative (1) of cyanoginosin-LA.

Ref: (Botes et al., 1984)

- i. Spectroscopic - Circular Dichroism - (N.A.)
- j. Solubility - (N.A.)
- k. X-ray Crystallography - (N.A.)
- l. Crystal Description - (N.A.)
- m. Specific Gravity - (N.A.)

- n. Misc. Properties - Stable at basic pH: slowly degrades in acid environment; soluble in methanol, water, ethanol.

5. Chemistry:

- a. Derivatives - (N.A.)
- b. Chemical Synthesis - (N.A.)

6. Production:

Microcystis aeruginosa Witbank Dam strain as well as all other strains in culture are grown in batch culture using about 3 different inorganic mineral media. For the Witbank strain a modified medium of Volk and Phinney (1968) was used (Botes et al., 1982). Other mineral media include ASM-1 (Carmichael, 1985B; Gorham et al., 1964) or BG-11 (Carmichael, 1985B). BG-11 is listed as #616 in the American Type Culture Collection Catalogue of Strains I (1982). Its composition (g/l) is as follows:  $\text{NaNO}_3$  = 1.5;  $\text{K}_2\text{HPO}_4$  = 0.04;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  = 0.075;  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  = 0.036; citric acid = 0.006; ferric ammonium citrate = 0.006; EDTA (Disodium salt) = 0.001;  $\text{Na}_2\text{CO}_3$  = 0.02; 1 ml/liter of trace metal mix A5. Trace metal mix A5 has a composition of:  $\text{H}_3\text{BO}_3$  = 2.86 g;  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  = 1.81 g;  $\text{ZnSO}_4$  = 0.222 g;  $\text{Na}_2\text{MoO}_4$  = 0.39 g;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  = 0.079 g;  $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$  = 49.4 mg; distilled water = 1.0 liter. The pH should be about 7.1 after sterilization.

7. Analytical Procedures:

- a. Biological - Assay for toxicity (of field bloom, cultured cells or toxin extracts) is by mouse bioassay (intraperitoneal injection). Signs of poisoning are monitored over a 3-4 hour period and compared with the gross symptomology listed in 9.c.

Recently a monoclonal antibody has been developed against cyanoginosin-LA. It also appears to react with at least some of the other amino acid variant peptide toxins (Kfir et al., 1985). It is proposed that this antibody be used to detect toxins in water supplies and to help examine the cellular effects of the toxin.

- b. Extraction and Purification - The extraction of the peptide toxin from lyophilized cells is listed below. The Bond Elute C-18 column is used to remove photosynthetic pigments before elution on a G-25 column (Carmichael, unpublished data).

## MICROCYSTIN

1 gm cells + 200 ml 5% butanol -20% methanol-75% water

Stir 1-3 hours at 4°C

Centrifuge 4750 G one hour

Repeat 3X with cell pellet

Combine supernatants

Reduce volume to 300-350 ml by air drying

Supernatant passed through analytical bond elute C-18 column

Elute toxic fraction with 3-5 ml 100% MeOH

Repeat process 3-4X

Dry combined methanol extract with nitrogen

Dissolve residue in 5 ml water

Pass through 3.0 micron millipore filter

K-26 pharmacia column (26 mm x 80 cm)

With 100 gm sephadex G-25

Elute in 5% methanol water

Monitor at 240 nm

Toxin is first large peak off the column

HPLC-Altex C-18 9.4 mm x 25 cm

0.01 ammonium acetate in 26% acetonitrile/water

Flow rate 3 ml/min

Monitor at 240 nm

(Toxic peaks elute in 10-20 minutes)

Lyophilize toxic peak

Desalt toxin by HPLC

As in preceding step - using 26% acetonitrile/water

Store toxin at -80°C until use

8. Biosynthesis - (N.A.)

9. Toxicology:

a. Sensitive Hosts - In vertebrates these peptide toxins probably exert their effect on the liver system. Most economic losses are due to domestic and wild animal losses from consumption of toxic cells and extracellular toxin in the water supply. Many temperate latitude countries have documented toxic blooms (Carmichael, 1985A).

b. LD<sub>50</sub> (intraperitoneal mouse) for purified peptide toxins are all about 50 ug/kg. The LD<sub>50</sub> ug/kg. The LD<sub>50</sub> is the same in rats but survival times at the minimum lethal dose can be 24-40 hours whereas in mice it is 1-2 hours. No oral LD<sub>50</sub> has been established in mice or rats but is estimated in sheep to be about



1000 mg/kg using toxic cellular bloom material (Jackson et al., 1984).

c-d. Gross Symptomology/Pathology - The consistent pathological findings in animals poisoned by Microcystis cells or purified toxin include the following:

- 1) Death within 1-3 hours
- 2) Livers swollen, blood engorged, liver weight 2-3X that of control animals
- 3) Microscopic examination of the liver reveals centrilobular hemorrhagic necrosis with loss of characteristic hepatic plates.

All other organs appear normal except the lung which is mildly congested with occasional intravascular protein deposits, described as atypical granular thrombi. Cause of death has been proposed to be either shock resulting from direct hepatocellular damage and massive hemorrhage of the liver; or right heart failure due to pulmonary congestion from the atypical thrombi (Slatkin et al., 1983; Theiss and Carmichael, 1985).

e. Molecular Basis of Action - (N.A.)

f. Synergism Data - (N.A.)

g. Structure: Function Studies - (N.A.)

10. Detoxification/Decontamination - (N.A.)

11. Microcystis peptide toxins when purified and lyophilized are white crystalline powders, which are mildly hygroscopic and electrostatic. They are stable under alkaline conditions and room temperature for at least 2 years. Spills should be cleaned up with aqueous/methanol washes at acid pH.

12. References:

1. Bishop, C.T., E.F.L.J. Anet, and P.R. Gorham, Isolation and identification of the fast-death factor in Microcystis aeruginosa NRC-1. Can. J. Biochem. Physiol. 37: 453-471, 1959.
2. Botes, D.P., H. Kruger, and C.C. Viljoen, Isolation and characterization of four toxins from the blue-green algae, Microcystis aeruginosa. Toxicon 20: 945-954, 1982.
3. Botes, D.P., A.A. Tuinman, P.L. Wessels, C.C. Viljoen, H. Krueger, D.H. Williams, S. Santikarn, R.J. Smith, and S.J. Hammond, The structure of cyanoginosin-LA a cyclic heptapeptide toxin from the cyanobacterium Microcystis

- aeruginosa. J. Chem. Soc. Perkin. Trans. 1: 2311-2318, 1984.
4. Carmichael, W.W. Algal Toxins. In: Callow, J.A. (ed.) Advances in Botanical Research, Vol. 12. Academic Press, London, p. 47-101, 1985A.
  5. Carmichael, W.W. Isolation, culture and toxicity testing of toxic freshwater cyanobacteria (blue-green algae). Proc. ISHC Symposium, Leningrad, USSR, Sept. 1984. Gordon & Breach, New York (In Press), 1985B.
  6. Gorham, P.R., J. McLachlan, U.T. Hammer, and W.K. Kim, Isolation and culture of toxic strains of Anabaena flos-aquae (Lyngb.) de Breb. Verh. Int. Verein. Limnol. 15: 796-804, 1964.
  7. Jackson, A.R.B., A. McInnes, I.R. Falconer, and M.T.C. Runnegar, Clinical and pathological changes in sheep experimentally poisoned by the blue-green alga Microcystis aeruginosa. Vet. Pathol. 21: 102-113, 1984.
  8. Kfir, R., E. Johannsen, and D.P. Botes. Preparation of anti-cyanoginosin-IA monoclonal antibody. In: Proc. 6th Int. Conf. Mycotoxins and Phycotoxins, Pretoria, Republic of South Africa, August 1985. Elsevier, Amsterdam (In Press), 1985.
  9. Runnegar, M.T.C. and I.R. Falconer. Isolation, characterization and pathology of the toxin from the blue-green alga Microcystis aeruginosa. In: Carmichael, W.W. (ed.) The Water Environment: Algal Toxins and Health. Plenum Press, New York, p. 325-342, 1981.
  10. Santikarn, S., D.H. Williams, R.J. Smith, S.J. Hammond, D.P. Botes, A. Tuinman, P.L. Wessels, C.C. Viljoen, and H. Kruger, A partial structure for the toxin BE-4 from the blue-green algae, Microcystis aeruginosa. J. Chem. Soc. Chem. Commun. 12: 652-654, 1983.
  11. Scott, W.E., the isolation of Microcystis. S. Afr. J. Sci. 70: 179, 1974.
  12. Siegelman, H.W., W.H. Adams, R.D. Stoner, and D.N. Slatkin, Toxins of Microcystis aeruginosa and their hematological and histopathological effects. In: Ragelis, E.P. (ed.) Seafood Toxins. American Chemical Society, Washington, D.C., p. 407-413, 1984.
  13. Slatkin, D.N., R.D. Stoner, W.H. Adams, J.H. Kycia, and H.W. Siegelman, Atypical pulmonary thrombosis caused by a toxic cyanobacterial peptide. Science 220: 1383-1385,

1983.

14. Theiss, W.C. and W.W. Carmichael. Physiological effect of a peptide toxin produced by the freshwater cyanobacteria (blue-green alga) Microcystis aeruginosa strain 7820. In: Steyn, P.S. and Vleggaar R. (eds.) Mycotoxins and Phycotoxins. Elsevier Science Publ. Amsterdam, p. 353-364, 1986.
15. Volk, S.L. and H.K. Phinney, Mineral requirements for the growth of Anaebaena spiroides in vitro. Can. J. Bot. 46: 619-630, 1968.

**DISTRIBUTION**

1 COPY	COMMANDER U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND ATTN: SGRD-RMI-S FT. DETRICK FREDERICK, MD 21701-5012
5 COPIES	COMMANDER U.S. ARMY MEDICAL RESEARCH INSTITUTE OF INFECTIOUS DISEASES ATTN: SGRD-U12-M FORT DETRICK FREDERICK, MD 21701-5011
12 COPIES	ADMINISTRATOR DEFENSE TECHNICAL INFORMATION CENTER ATTN: DTIC-DDAC CAMERON STATION ALEXANDRIA, VA 22304-6145
1 COPY	DEAN, SCHOOL OF MEDICINE UNIFORMED SERVICES UNIV. OF THE HEALTH SCIENCES 4301 JONES BRIDGE RD. BETHESDA, MD 20814-4799
1 COPY	COMMANDANT ACADEMY OF HEALTH SCIENCES, US ARMY ATTN: AHS-CDM FT. SAM HOUSTON, TX 78234-6100